THE UNIVERSITY OF KASHMIR

IQBAL LIBRARY

244

DATE LABEL

19 AUG 1971			20 JAN 2002
27 OCT 1976			
10 JUN 1984			12 JUL 1988

Call No. .....

Acc. No. 13013 Date .....

J. & K. UNIVERSITY LIBRARY

This book should be returned on or before the last date stamped above. An over-due charge of .06 P. will be levied for each day, if the book is kept beyond that day.

INDUSTRIAL MICROBIOLOGY

INDUSTRIAL MICROBIOLOGY

By

SAMUEL CATE PRESCOTT, Sc.D.

Professor of Industrial Biology (Emeritus), Former Head of the Department of Biology and Public Health and Dean of the School of Science, Massachusetts Institute of Technology

and

CECIL GORDON DUNN, PH.D.

Associate Professor of Industrial Microbiology in the Department of Food Technology, Massachusetts Institute of Technology

QW-3449
P93
113-127.

SECOND EDITION

McGRAW-HILL BOOK COMPANY, INC.

NEW YORK TORONTO LONDON

1949

ALLAMA IQBAL LIBRARY
13013

INDUSTRIAL MICROBIOLOGY

Copyright, 1940, 1949, by the McGraw-Hill Book Company, Inc. Printed in the United States of America. All rights reserved. This book or parts thereof, may not be reproduced in any form without permission of the publishers.

QT 01
K61
JAMMU & KASHMIR UNIVERSITY
LIBRARY
No. 13013
Date 9.8.55
SRINAGAR
CHECKED
576
P92A1

BY THE MAPLE PRESS COMPANY, YORK, PA.

PREFACE TO THE SECOND EDITION

Since the publication of the first edition of this work in 1940, marked advances have been made in the subject by numerous investigators in many countries. The increase in interest in this field of microbiology which has thus been demonstrated and the wide range of the researches which have been carried on supply abundant proof of the recognition of the potential importance of fermentations and related processes in industrial biochemical operations. In this edition we have tried to keep pace with this gratifying extension of knowledge and to review it now in a form which may be useful to students of this branch of technology and to those concerned with research or technical application therein.

The inclusion of all this new material has made it desirable to revise and consolidate much of the earlier material and to present in the tables relating to production statistics, fermentation products, etc., the latest available data. The chapters on yeast have been rewritten into a single chapter and expanded by much new material on food and fodder yeasts, riboflavin production, and discussion of methods of operation industrially.

Five new chapters have been added, giving extended descriptions of new processes of saccharification, recent work on yeast production and yeast products, the production and properties of 2,3-butanediol, the itaconic and related fermentations, and comprehensive discussion of antibiotics. All these have been prepared with special reference to industrial applications and the possibilities of future developments.

The revision of a book in a field showing such rapid development has of necessity increased the volume of material greatly, in this case about 50 per cent. There were 60 illustrations in the first edition, and the present volume contains 124.

Grateful acknowledgment is made to the many authors and publishers who have kindly permitted use of quoted material, photographs, cuts, and tables.

We extend our thanks to the editors and publishers of scientific journals from which reproductions have been made, particularly to Dr. Walter J. Murphy, Editor of *Industrial and Engineering Chemistry;* the Williams and Wilkins Company, publishers of the *Journal of Bacteriology;* Dr. R. A. Bottomley, Managing Editor, *Cereal Chemistry;* Dr. Philip P. Gray, Wallerstein Laboratories; Dr. F. E. Nelson, Editor of the *Journal of Dairy Science;* and others. We are happy to acknowl-

edge the help received from various branches of the United States Department of Agriculture—from Dr. Kenneth Raper, Dr. Moyer, Dr. Lockwood, and their associates of the Fermentation Division, Northern Regional Research Laboratory; from Dr. Harry Humfeld and others of the Western Regional Research Laboratory; from Dr. Morrison Rogosa, Bureau of Dairy Industry; from Dr. Edward G. Locke and Dr. Jerome F. Saeman of the Forest Service; and from Drs. Ivan D. Jones and John T. Etchells, Raleigh, N. C. We wish to thank our Canadian friends, especially Dr. G. A. Ledingham and his associates of the National Research Council of Canada and Dr. Katznelson and his collaborators of the Canada Department of Agriculture for the large amount of information supplied concerning the 2,3-butanediol fermentation. We are indebted to our colleagues in other colleges and universities—to Dr. L. A. Underkofler, Iowa State College, for making suggestions concerning the Chapter on "Saccharifying Agents: Methods of Production and Uses"; to Drs. M. J. Johnson, J. J. Stefaniak, and their associates of the University of Wisconsin; to Drs. W. V. Cruess and Emil Mrak and their colleagues of the University of California; to Dr. F. W. Fabian, Michigan State College; to Dr. Carl C. Lindegren of Southern Illinois University; to Dr. Selman A. Waksman of the New Jersey Agricultural Experiment Station; and to Dr. Carl F. Cori, Washington University. We wish to thank our friends in industry—Merck and Company; Drs. Paul Kolachov and G. W. Packowski of Joseph E. Seagram & Sons, Inc.; Drs. H. Kothe and F. R. Swift, The Fleischmann Laboratories; Dr. George A. Jeffreys of the Jeffreys Research & Development Co.; Dr. A. R. Stanley of Commercial Solvents Co.; Dr. T. H. M. Taylor, formerly of the Cutter Laboratories; Dr. E. O. Ericsson, Puget Sound Pulp and Timber Co.; Schock, Gusmer & Co., Inc.; and the Calvert Distilling Co.—for supplying photographs or other illustrative material. We thank Prof. William L. Campbell, Head of the Department of Food Technology, and our other colleagues for their helpfulness. We wish to express our especial thanks and appreciation to Mrs. Cecil G. Dunn who painstakingly read the manuscript and the galley proofs and made many valuable suggestions. We wish to thank Miss Anita Frey and Miss Virginia Pochetti for their help in typing the manuscript.

SAMUEL C. PRESCOTT
CECIL G. DUNN

CAMBRIDGE, MASS.
July, 1949

PREFACE TO THE FIRST EDITION

For hundreds or even thousands of years mankind has practiced domestic arts in which microbes are the invisible but active agencies of desired change. Wine making, vinegar production, brewing, and the making of leavened bread were processes known to ancient peoples. Even when some of these processes became established on a larger scale the success was still somewhat dependent on chance and the combination of fortuitous circumstances with a degree of skill born of experience. Not until less than a hundred years ago was there any scientific appreciation of the real part microbes play in the transformations of organic matter.

Industrial microbiology is one of the important outgrowths of those fundamental researches conducted by Pasteur which have made his name the most highly revered in the whole realm of microbiology. Although his preeminence has been especially recognized in another field of microbic investigation which has greatly promoted man's welfare, the bacteriology of infectious disease, it should not be forgotten that his first studies were in fermentation and were conducted in aid of industries. He may thus especially be regarded as the founder of industrial microbiology as well as of medical bacteriology and immunology. Although the development of industrial microbiology was slow during the quarter century following Pasteur's work, a few outstanding contributions, such as those of E. C. Hansen, Jorgensen, Lafar, Delbruck, Duclaux, Lindner, and others, added materially to the subject before the opening of the present century. Since that time, development has been much more rapid, and industrial microbiology has now become a large and widely recognized field of study and practical application. In 1896 the senior author organized in the Biological Department at the Massachusetts Institute of Technology the first course of classroom instruction in industrial biology given in America. Of relatively small scope at the beginning, the course has been expanded from time to time as the subject has grown in importance, and the present related and parallel courses in industrial microbiology and food technology are outgrowths of its development.

The authors have prepared the present volume, believing that a comprehensive work in English dealing with this subject and presenting both theoretical and practical aspects of fermentation would be useful. Whether used primarily as a text for fairly advanced students or as a work

of reference, it is assumed in advance that the student or reader will already have had somewhat extensive training in biology and general bacteriology, and at least the fundamentals of organic chemistry and biochemistry. For this reason the general description and taxonomic treatment of the great class of the bacteria have been limited to the groups intimately concerned with the processes to be discussed later.

It is the purpose of this volume to outline, in a concise but comprehensive manner for students thus prepared, the fundamentals of industrial microbiology, and to present descriptions of the more important processes within the field.

No single text of moderate size can give a complete and detailed treatment of every aspect of the subject. There are in this volume what may, in some quarters, be regarded as serious omissions. For example, discussion of the commercial manufacture of sera, vaccines, and other therapeutic agents is omitted, as is also reference to the production of cultures of nitrogen-fixing bacteria, and of butter- and cream-ripening organisms, etc., although in a limited way each of these might be regarded as falling within the scope of industrial bacteriology. The reasons for these omissions will, it is believed, be evident on further thought—the first subject is closely related to public health and medicine; and the second subject seems more properly to belong to agricultural or to dairy bacteriology.

The efforts of the authors have been especially directed (1) to a broad treatment of those large-scale fermentations in which the end products are of themselves industrially or potentially important, and (2) to consideration of others that are of special significance because of the modifications of the quality of substrate materials that they produce, as in some aspects of food technology.

The various alcoholic fermentations as applied in brewing, wine-making, and distillation industries; the acetic fermentation; the lactic fermentation; and the fermentations yielding butyl alcohol, acetone, citric acid, glycerol, gluconic acid, etc., are described and discussed since they represent type processes that illustrate basic principles and may supply the key to modes of inquiry that may be useful to the student in later researches or in the pursuit of new aspects of industrial fermentations.

A more comprehensive treatment of the general aspects of the subject will be found in the introductory chapter.

Consideration is also given to the discussion of some of the more recently discovered fermentations which have been investigated and which seem to offer possibilities of industrial significance in the near future. These are treated not only from the standpoint of the organisms

involved but also with reference to the raw materials and production methods that may be employed and to the biochemistry of the reactions catalyzed by the microbes.

The work has attempted to present the subject in a unified way, although it divides somewhat naturally into four parts, the first three dealing, respectively, with the biochemical activities of yeasts, of bacteria, and of molds. Within each of these three classes of microorganisms with their extremely numerous and varied groups and species are found relatively few types having marked activity as causative agents of fermentation, but these are important since they represent types which probably can be employed economically and in large-scale operations. The last part of the book deals with specialized activities of microorganisms and some higher types of organisms in relation to undesirable changes produced in textile fibers, in wood itself, and in structures built of wood. Two appendices treat briefly of subjects useful to the industrial microbiologist. The first deals with the control of microbes by the use of germicides, and the principles of testing disinfectants, and the second with the treatment and disposal of industrial microbiological wastes.

At the end of each chapter, references are given, in order that the student who desires may continue his study of the subject by consulting original papers. The subject has now become so voluminous that it is impossible to present complete bibliographies, and although the references presented are regarded as the most useful for the student, it may be that numerous important publications have not been mentioned here.

The authors wish most gratefully to acknowledge their indebtedness to many who have assisted in the preparation of this book by permission to use tables, plates, and other illustrative material, and to others who have aided by the careful reading of some of the chapters. Special thanks are due Prof. W. H. Peterson and his associates at the University of Wisconsin; to Prof. C. H. Werkman and his colleagues at Iowa State College; to Drs. H. S. Knight, O. E. May, H. T. Herrick, A. J. Moyer, L. B. Lockwood, and their associates in the U.S. Department of Agriculture; to Dr. W. V. Cruess of the University of California; and to the editors of various journals, especially Dr. L. V. Burton and H. E. Howe, who have kindly given permission to use tables and figures. The authors are most appreciative of the kindness of Drs. G. B. Sippel and W. C. Tobie for their careful reading of the chapter on Brewing and the section on Rum Manufacture, respectively. Others to whom the authors wish to express gratitude are Dr. J. W. Lawrie of the Jos. Schlitz Brewing Company, Dr. C. N. Frey of the Fleischmann Laboratories, Dr. G. O. Lines of the Commercial Solvents Corporation, Dr. R. Schwarz of Schwarz Laboratories, and to their European fellow workers, Dr. A. J. Kluyver,

Dr. A. Guilliermond, Dr. Ö. Winge, Dr. O. Meyerhof, Dr. H. Raistrick, Dr. C. G. Anderson, and Dr. Marjory Stephenson, for their friendly cooperation in supplying photographs, tabular material, and other data. Finally, the authors acknowledge with thanks the cooperation of their departmental colleagues, of Mrs. Cecil G. Dunn, who has made many suggestions and given unlimited cooperation in reading the proofs, and of Miss Janet Hartwell, who has rendered exceptional service in the preparation of the manuscript.

SAMUEL C. PRESCOTT
CECIL G. DUNN

CAMBRIDGE, MASS.
May, 1940

CONTENTS

PREFACE TO THE SECOND EDITION v

PREFACE TO THE FIRST EDITION vii

CHAPTER

I. INTRODUCTION 1

II. THE YEASTS 11

III. SACCHARIFYING AGENTS: METHODS OF PRODUCTION AND USES . . . 60

IV. THE PRODUCTION OF INDUSTRIAL ALCOHOL BY FERMENTATION . . . 113

V. FACTS AND THEORIES CONCERNING THE MECHANISM OF THE ETHYL ALCOHOL FERMENTATION 145

VI. BREWING 167

VII. WINE 194

VIII. THE DISTILLING INDUSTRIES 222

IX. PRODUCTION OF YEAST AND YEAST PRODUCTS 240

X. THE GLYCEROL FERMENTATION 299

XI. THE BACTERIA 309

XII. THE ACETONE-BUTANOL FERMENTATION 312

XIII. THE ACETONE-ETHANOL FERMENTATION 352

XIV. THE BUTYL ALCOHOL-ISOPROPYL ALCOHOL FERMENTATION 360

XV. THE ACETIC ACID BACTERIA AND SOME OF THEIR BIOCHEMICAL ACTIVITIES 370

XVI. THE PRODUCTION OF LACTIC ACID BY FERMENTATION 404

XVII. SAUERKRAUT 429

XVIII. PICKLES 439

XIX. SOME LACTIC ACID BACTERIA AND SOME FERMENTED MILK PRODUCTS 462

XX. CHEESE 468

XXI. THE PROPIONIC ACID FERMENTATION 477

XXII. THE PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL 487

XXIII. SOME MINOR BACTERIAL ACTIVITIES WITH INDUSTRIAL IMPLICATIONS 524

XXIV. THE MOLDS 553

XXV. THE CITRIC ACID FERMENTATION 572

XXVI. THE GLUCONIC ACID FERMENTATION 606

XXVII. THE FUMARIC ACID FERMENTATION 625

CHAPTER

XXVIII. THE GALLIC ACID FERMENTATION 630

XXIX. THE ITACONIC AND ITATARTARIC ACID FERMENTATIONS 632

XXX. THE KOJIC ACID FERMENTATION 644

XXXI. THE PRODUCTION OF LACTIC ACID BY MOLDS 653

XXXII. MANNITOL PRODUCTION BY MOLDS 661

XXXIII. MOLD ENZYME PREPARATIONS: USES AND PRODUCTS 664

XXXIV. THE PRODUCTION OF FAT BY MOLDS 676

XXXV. SOME MINOR CHEMICAL ACTIVITIES OF THE LOWER FUNGI 686

XXXVI. ANTIBIOTICS 708

XXXVII. TEXTILE MICROBIOLOGY 809

XXXVIII. THE MICROBIOLOGY OF WOOD 832

APPENDIX A. DETERGENCY, DISINFECTION, AND STERILIZATION 847

APPENDIX B. THE TREATMENT AND DISPOSAL OF INDUSTRIAL MICROBIOLOGICAL WASTES 858

INDEX 865

CHAPTER I

INTRODUCTION

Industrial microbiology is that portion of microbiological science which deals with the possible utilization of microorganisms in industrial processes, or in processes in which their activities may become of industrial or technical significance. Obviously the term "microbiology" in its broadest sense comprises that division of biological science which treats of the extremely small organisms of both plant and animal natures that Sédillot in 1878 grouped together under the inclusive term "microbes." Although this word is now often used synonymously with bacteria it is not so limited, since within its field may actually be included all types of very small living things, such as the protozoa, spirochetes, and minute parasitic worms, as well as those low types of fungi commonly spoken of as the "yeasts" and "yeast-like organisms," the somewhat indefinite group called "molds," and the extremely numerous and widespread group of the bacteria. The modern general use of the term "protozoology," which has resulted from the many careful studies of the lowest group of animal organisms, has to a great extent in recent years transferred the interest and extended descriptions of many of the types of the animal group of *Protozoa* from the general field of microbiology to this more specialized domain of study. A modern tendency apparently is to confine the term "microbiology" largely to organisms that fall within the realm of the lowest groups of botanical or plant life. It is in this sense that the word is used in this book.

For several decades it has been known that numerous kinds of yeasts, molds and other low fungi, and several types or groups of bacteria have direct relation, either favorable or unfavorable, to certain types of economic processes carried out in connection with industrial or factory operations such as brewing, wine making, cheese making, etc., which have grown up from small-scale or family arts. Knowledge in this field has greatly extended in recent years, as research in many countries and in many lines has been carried out. The scale of operations has greatly enlarged and concentrated in manufacturing plants. Industries have replaced household arts. These changes have made it evident that industrial microbiology is not only an exceedingly interesting branch of study but that it has already become a distinctly important branch of applied science, and one with even greater potentialities.

In any broad-gauge discussion of industrial microbiology it should be emphasized at the outset that knowledge in this field is of usefulness and significance from two diametrically opposite angles of application. On the first and more positive side, it is clearly advantageous to know the biological and biochemical characteristics of the many types of organisms that are the prime and direct causes of chemical transformation of materials into desired products. Here one utilizes the microorganisms capable of producing, by fermentation processes, relatively large quantities of chemical substances of usefulness and economic value. In this type of process the biochemical methods are in some instances the only economic methods of production. The manufacture of industrial alcohol and other industries based on alcoholic fermentation may be taken as a type of this class. Of equal interest to the microbiologist is the part played by organisms of these groups in composite operations, where the action sought is the production of somewhat smaller quantities of desirable by-products that should occur only as minor but important components in the final product. This is the aim in some branches of food manufacturing and in other commodity products.

On the other hand, it is recognized that fermentations incited by microbes may not always be desirable but may be quite the reverse. Competition by invading contaminants may wreck what would otherwise be a successful process. It is, then, of much importance to have knowledge of the organisms specifically useful in a process, and to be equally informed regarding those which oftentimes are destructive to or detrimental in manufacturing processes and are the causes of economic loss. The trained industrial microbiologist must be able to discover them, to recognize the type of damage they produce, and to become versed in the methods of combating them.

With all these viewpoints in mind, it is clear that industrial microbiology should include in its scope the study (I) of the numerous fermentation processes in which the production of alcohols, organic acids, glycerol, acetone, and other substances are end products; (II) of certain aspects of food-manufacturing processes, such as baking and the making of cheeses, butter, sauerkraut, and pickles, in which microbic agencies take a significant and important accessory part; (III) of food-conservation methods, such as canning and preserving, refrigeration, quick freezing, and drying, where sterilization or inhibition is imperative; and (IV) of the microbiological problems concerned with textile and commercial fibers. Obviously in the processes grouped under (II) and (III) and in a part of those under (I) industrial microbiology is intimately associated with food technology. The work of the industrial microbiologist may also deal with the production and technical uses of bacterial and fungus enzymes,

such as amylases and proteases, or with the preservation or protection of wood and the processes of commercial disinfection, wherein the application of suitable chemicals to restrain or prevent fungus, bacterial, or enzyme activity on walls and floors or on materials undergoing processing in the mill or manufacturing plant may make the difference between success and failure of operation.

The production of sera, vaccines, and other therapeutic agents commercially, although generally regarded as in the field of public health or medicine, and hence not here treated, might in reality be considered as a group of processes in industrial biology. It has seemed best to omit these processes in this volume, and also not to include the study of soil microbiology, the use of nitrogen-fixing bacteria, and the study of the phytopathology of economic crop plants, use of disinfectant or inhibitive sprays, etc. Although all these aspects of microbiology are extremely important in agriculture and therefore constitute a part of economic microbiology, they do not fall within the limits of this work.

In the present volume, industrial microbiology will be regarded essentially as the science and art of investigating and controlling technical fermentations, that is, of using microorganisms as reagents to produce desirable end products having possible or well-defined industrial uses and applications. Obviously, conditions in which losses due to contaminations or wholesale microbic infection affect manufacturing operations must enter into the subject matter. Although all the minute details of particular industries cannot be dealt with in a text of this scope, an attempt is made to present the principles and the general methods of operation. In addition to the consideration of the organisms and biochemical reactions concerned in the better-known industrial fermentations now in use, a few fermentations of present theoretical interest but perhaps potentially capable of technical development are also discussed.

It is important not to make too broad assumptions regarding industrial uses of microbes and to keep in mind the special qualifications which must be possessed by organisms in order that they may be economically utilized in the direct industrial production of materials having distinct commercial value. Obviously the number of species having this valuable property must be limited. The industrially important microbes may be characterized as having at least three outstanding qualities:

1. The ability to grow rapidly in suitable organic substrates and to be easily cultivated in large quantity.
2. The ability to maintain physiological constancy under these conditions and to produce the necessary enzymes readily and profusely, in order to bring about the desired chemical changes.
3. The ability to carry out these transformations under comparatively

simple and workable modifications of environmental conditions; and, since the reactions are exothermic, without the application of large quantities of external energy.

Obviously, industrial operations with microbes are more complicated than laboratory experiments but involve the same principles. Large-scale operations must include protection from contamination, and the chemical engineering equipments must be carefully designed to meet the special conditions required in each type of fermentation.

Hundreds of microbes can grow rapidly in solutions of organic substances without yielding significant quantities of valuable products. They may bring about decompositions that conform to the broad general definitions of a fermentation that will be set up, but they may not be productive of a technically important or industrial fermentation.

Microorganisms of Fermentation.—As has been intimated, the microorganisms of fermentation include yeasts, molds, and bacteria. These microorganisms are unable to manufacture their own food by the ordinary process of photosynthesis since they lack chlorophyll and are classified as fungi, belonging to the phylum *Thallophyta*. Certain of the *Ascomycetes* (sac-fungi) and of the *Phycomycetes* (alga-like fungi), and a large number of species of the bacteria are the principal microorganisms that are directly concerned. A few of the *Basidiomycetes* are of significance in special types of breakdown of wood and fibers and are thus productive of changes comparable to fermentations.

The microorganisms of fermentation differ widely in respect to morphology, size, reaction to free oxygen, manners of reproduction, growth requirements, ability to assimilate or ferment raw (natural) substances, and in other ways. But they are similar in that they are "colorless" and grow most actively in darkness or diffused light, and all produce enzymes by which they catalyze the reactions ascribed to them.

Variation in Strains.—Even in a so-called "species" there may be a large number of types or strains, and, even in a so-called "pure-strain," variation may occur under different environmental and nutritional conditions. Although the usual mode of increase by asexual reproduction favors the constancy of a given species, many factors may tend to cause changes in the chemical composition of a microorganism, the type and quantity of end products formed, and the rate of growth and reproduction. For example, the nature and quantity of the nutrient substances supplied, the temperature of incubation, the reaction of the medium, the oxygen relationships, the presence or absence of stimulating or inhibiting substances in the medium, and various other factors must be controlled and made similar in order to obtain analogous results with the same or related strains of a microorganism. The importance of mere traces of a sub-

stance cannot be too much stressed in some instances. Some of the apparent differences in results obtained in research in different laboratories with the supposedly same strain of organism may disappear when conditions of culture and the chemical composition of the media become exactly or essentially the same. It is, however, probably impossible to maintain perfect lack of variation over long periods.

Although some differences are only apparent ones, frequently true biologic variations exist in strains. Too much work has been carried out by responsible laboratories to leave any doubt as to this fact. For example, some strains of a microorganism apparently require added growth-accessory substances, while other strains require none; some strains of *Aspergillus niger* are stimulated by iron and zinc salts, others receive no apparent stimulation from these salts.

Fermentation.—From the biochemical standpoint, fermentation is the name given to the general class of chemical changes or decompositions produced in organic substrates through the activity of living microorganisms. Thus there may be many kinds of fermentation falling within this category depending on the type of organism involved, the type of substrate, or even the conditions imposed, such as pH, or oxygen supply. The word "fermentation" is a term that has undergone numerous changes in meaning during the past hundred years. According to the derivation of the term, it signifies merely a gentle bubbling or "boiling" condition, and the term was first applied when the only known reaction of this kind was in the production of wine. Even then no knowledge existed as to the cause. Thus in an active ethyl alcohol fermentation, as in a wine or cider fermentation, carbon dioxide is always liberated in the form of bubbles of gas, which at the height of the reaction may cause a marked agitation or movement of the liquid medium, especially in a large vat or tank, sufficient to give to it the appearance of a boiling liquid. This interpretation of the word was the accepted one for several thousand years. After Gay-Lussac studied the process the meaning was changed to signify the breakdown of sugar into alcohol and carbon dioxide. With the increase in knowledge following Pasteur's researches as to the *cause* of this change in the nature of the material fermenting, the word became associated with microorganisms and still later with enzymes, which are the biologically produced reagents by which microbes work. For a long time fermentation was especially associated with carbohydrates and, indeed, is often so considered at present, but a broader conception of these biologic reactions seems to be more logical. Thus putrefaction and the breakdown of fats by microbes are to be regarded as special kinds of fermentations.

Although fermentation is frequently or even generally associated with

the evolution of gas due to the action of living cells, neither gas evolution nor the visible presence of living cells is today regarded as an essential criterion of fermentation. In certain fermentations, for example, some of the lactic fermentations, no gas is liberated. Again, fermentation might result (although it is not frequently the case) from the use of cell-free enzyme extracts that for a time catalyze the reactions. Gas can be liberated in some of these processes, as for example with a zymase preparation. Cell-free fermentations are, however, unknown in nature and when produced in the laboratory are considerably slower than fermentations where the living cells are present, and are largely of theoretical interest.

Fermentations are so varied in character that any broad definition that will cover this whole class of biochemical processes must be couched in very general terms. Nevertheless, it is sometimes useful to gain a briefly stated concept of such a group of reactions. The idea might be expressed as follows:

A fermentation, in the broad sense in which the term is now used, may be defined as a process in which chemical changes are brought about in an organic substrate, whether carbohydrate or protein or fat or some other type of organic material, through the action of the biochemical catalysts known as "enzymes," elaborated by specific types of living microorganisms.

For more exact characterization, the kind of fermentation, such as alcoholic, lactic, acetic, etc., must be specified. Since the majority of fermentation processes first studied concerned carbohydrates, these are often regarded as the essential materials, but it must be clear that proteins, fats, some salts of organic acids, and alcohols can be broken down by similar agencies and thus fall within the classification of fermentable material.

Enzymes.—The student of biology or biochemistry will already have learned of the universal association of enzymes with living matter and the important part played by them in all types of vital activity. The activities of enzymes are especially prominent in the phenomena of digestion in animals, in the transformations of starch in sprouting seeds, etc. These and many other observations lead to the view that enzymes are the essential organic catalysts, possessed or produced by all living cells, without which the processes of life would cease or be impossible. It is, furthermore, well established that these enzymes are of two types, called "exoenzymes" and "endoenzymes," according to their sphere of activity outside or within the confines of the cells that elaborate them. Both types are extremely important. Exoenzymes, liberated by the manufacturing cells, penetrate and break down the organic materials

outside the cell, such as the proteins, starches, and fats of food materials, giving rise to soluble derivatives and so making it possible for the products of their activity to be absorbed through the cell membrane. Energy liberated by the enzyme action outside the cell is of comparatively little value to the cell. In general it is found that exoenzymes liberate relatively little energy, as heat, especially in the most commonly produced hydrolytic processes.

The endoenzymes elaborated and retained within the living cell, on the other hand, behave quite differently. The food substances, having been absorbed into the cell, may be further transformed and broken down by the action of the endoenzymes, and this process is accompanied by the liberation of relatively large amounts of energy, this energy being available to the cell.

The microorganisms of fermentation are notable for their ability to produce enzymes of both these classes. Molds, yeasts, and bacteria can secrete or elaborate a wide variety of enzymes, possibly of a greater range than any other single cells, since all the functions of growth, reproduction, digestion, assimilation, etc., that are distributed among the various tissues and organs of higher plants and animals seem here to be concentrated in the single minute cell. This may explain why it is that, in these low groups of extremely small plants which are essentially unicellular in structure, we find a higher degree of enzyme productivity and of fermentative capacity than is exhibited elsewhere in the world of living things.

The enzymes are therefore the reactive substances or catalyzers that microorganisms employ in bringing about the specific changes or fermentations that are characteristic of different species or groups of microbes. One can visualize each strain, species, or genus as having its own armamentarium of enzymes or its own peculiar ability to secrete them. In this individual potentiality may lie one of the factors that account for the variations in strains mentioned earlier in this chapter.

TABLE 1.—ENERGY LIBERATED FROM ONE GRAM OF SUBSTRATE BY ENZYMES[1]

Exoenzymes	Calories	Endoenzymes	Calories
Pepsin	0	Lactacidase	82
Trypsin	0	Alcoholase	149.5
Rennet	0	Urease	239
Lipase	4	Oxidase (vinegar)	2,530
Invertase	9.3		
Maltase	10		
Lactase	23		

[1] ANDERSON, C. G., "An Introduction to Bacteriological Chemistry," Williams, Wood & Company, The Williams & Wilkins Company, Baltimore, 1938.

Energy Relationships.—The preceding table, which shows the energy liberated from 1 g. of substrate by enzymes, will give some insight into the relative energy values of exoenzymes and endoenzymes.

The question naturally arises, does the microorganism have a requirement for a major portion of the large amounts of energy liberated within the cell, or is much of the energy liberated merely due to the great excess of enzyme product or the result of unrestricted enzyme action? Experiments with yeast cells indicate rather emphatically that the energy evolved is not a measure of metabolic requirements, but is the result of enzymes acting on an abundant and suitable substrate. However, further experimentation is necessary to lend additional weight to this suggestion. Whatever the theoretical aspects, it is known in practice that under the artificial conditions of a great volume of fermenting liquid, in which billions of cells are active, energy in the form of heat may be liberated to a marked degree. In such large masses of fermenting material where the heat produced cannot be readily diffused or conducted away the rise of temperature may become so great as to impair the organisms that produce the reacting catalyst. In vinegar fermentation the heat energy evolved may be sufficient to cause a rise of temperature that may inhibit normal cell growth and stop further activity.

Intense Activity of Microorganisms.—Many microorganisms, in comparison to the higher organisms, are intensely active in respect to the chemical changes that they bring about. The examples cited above show this fact and also are typical of the fact that all fermentative processes are exothermic. Burchard[1] has calculated that 1 g. (wet weight) of *Micrococcus ureae* decomposes 180 to 1,200 g. of urea per hour, while Haacke estimated that 1 g. (wet weight) of a lactose fermenting organism breaks down 178 to 14,890 g. of lactose in 1 hr. Even though these calculations may be of only approximate value, the enormous activity of microorganisms is evident. This great chemical activity of microorganisms is associated with their simple life requirements, the ease with which they attack food for energy, the rapidity of growth or reproduction, and possibly their capacity for maintenance under different conditions.

Specific Types of Fermentative Change.—It has been intimated in the foregoing pages that the specific or characteristic kind of fermentation produced by an organism is dependent on its enzyme-producing powers. One might expect, therefore, that those organisms most nearly related generically would be most similar in their fermentation relationships. This seems to be generally the case, although it does not follow that organisms belonging to different biological groups are necessarily totally

[1] STEPHENSON, M., "Bacterial Metabolism," Longmans, Green & Company, London, 1930.

unlike in their ability to catalyze fermentation processes having some of the same end products. The "true" yeasts and some other budding fungi nearly related to them are the organisms that most commonly produce ethyl alcohol, and because of this fact it is often stated that the yeasts are the microorganisms of alcoholic fermentation, but there are many budding fungi, classified in groups or families morphologically very similar to yeasts, that apparently have no ability to produce ethyl alcoholic fermentation or any other with large quantities of a particular end product. On the other hand there are a few fungi, somewhat higher in the scale of organization than yeasts, that can produce ethyl alcohol under certain conditions of substrate, pH, relation to oxygen, etc. Even a very small number of species of bacteria can produce alcohol. These are unusual cases but apparently demonstrate the great versatility of the low colorless plants in developing their enzyme systems. In general, the molds that have marked fermentation power are producers of organic acids and of products of protein decomposition. Similarly, the fermenting types of bacteria commonly give rise to organic acids, frequently different from the acids produced by molds, and to higher alcohols than ethyl. It cannot be assumed that all species of microorganisms belonging to the three groups that have been mentioned as constituting the microorganisms of fermentation actually have marked ability in this respect. It would be more nearly correct to say that the significant fermentation organisms represent a minority of all the organisms so classed.

Energy Value of Substances.—The substances acted on by microorganisms present a dual role, the first as a food, the second as a material to be transformed through the action of enzymes that have been produced in excess of nutritional requirements. In general, the energy value of a substance, such as glucose, depends on the degree of oxidizability it can undergo. In the complete oxidation of glucose, as in burning, considerable energy is evolved. This is also true in the breakdown by aerobic organisms, while in the anaerobic breakdown of this sugar only a fraction of the potential energy is liberated. Consequently, in order to obtain an equivalent amount of energy, several times as much glucose must be broken down under anaerobic conditions as would be required under aerobic conditions.

The breakdown of nutrients in fermentation is not merely expressed in the evolution of heat but is in early stages at least always accompanied by division of new cells. For example, in the manufacture of compressed yeast, the nutrient medium is supplied not only with organic food but also with a very large amount of air. Aerobic oxidations are favored and under such conditions, along with the fulfillment of nitrogen and phosphate requirements, pH control, and the employment of a low concen-

tration of sugar, large yields of yeast cells are produced. Under essentially anaerobic conditions, such as would exist in the normal industrial fermentation for ethyl alcohol, much more sugar must be utilized to produce the same quantity of yeast cells, and a large amount of the organic matter of the substrate is converted to the characteristic end products, one of which, ethyl alcohol, is still comparatively high in potential energy.

It should now be clear that a fermentation process that may become of industrial significance is not a simple matter of inoculating organic matter with microbes that can derive food from it, but is instead the setting up of a system of biochemical reactions between a substrate and an organism that can partially decompose it in a special manner under controlled and workable conditions. The substrate must be abundant, inexpensive, and fairly high in potential energy. The organism must have ability to attack, by means of its enzymes, certain chemical groupings in the composition of the substrate and, by hydrolysis, oxidation or reduction, or other means, disrupt certain bonds in the substrate molecules and give rise to stable products under the imposed conditions. Thus by a single reaction or a series of coincident or successive enzyme reactions carried out under definite conditions it may bring about eventually a desired product or group of products of lower potential energy.

CHAPTER II

THE YEASTS

The yeasts and other yeast-like organisms, often grouped together under the name *Blastomycetes* or "budding fungi," belong to the subdivision of the thallophytes designated as the *Eumycetes*, or true fungi, since they possess no chlorophyll. All the organisms here grouped are unicellular plants of microscopic size and widely distributed in nature, and they occur especially in the top layers of the soil, in dust, and on the fruits and leaves of many plants. The grape, apple, pear, and many other fruits have these organisms almost constantly present, and the soil of orchards and vineyards where the microbes live over the winter is particularly well populated with yeast cells. Distribution is easily effected by wind and on the bodies of bees, wasps, and other insects.

The budding fungi may be separated into two rather unequal divisions: the spore-forming (sporogenous) or true yeasts represented by the family *Endomycetaceae* (*Saccharomycetaceae*); and the nonsporing (asporogenous) pseudo or false yeasts, which are represented by the families *Rhodotoraceae*, *Torulopsidaceae*, and *Nectaromycetaceae*. The true yeasts include about 17 genera and a large number of so-called "species," many of which in turn show numerous slightly different strains or "types." Although most taxonomists are in agreement as to the main groups, definite classification into genera and species is externally difficult and unsatisfactory from the botanical standpoint. Industrially, however, the one genus *Saccharomyces* is of outstanding interest, as most yeasts having any technical uses belong herein.

The yeasts grow most luxuriantly in solutions containing sugar and the other necessary food requirements. In a fluid nutrient medium the cells ordinarily occur singly or in twos or threes or attached to one another in small clusters that are actually groups of cells of different generations, as a result of the characteristic method of vegetative increase by budding. On agitation, these groups break apart, and most of the cells eventually settle to the bottom of the container as sedimentary cells. If the culture medium remains undisturbed, islands of cells or films of greater or less complexity may appear on the surface. Sometimes these superficial cells may appear as chains or filaments of cells, resembling hyphae; and often with a number of smaller cells budded off

at the nodes. Some true yeasts, such as those of the genus *Endomyces*, may form a true mycelium or mass of cells. On favorable solid media, compact colonies are produced, varying in size and surface markings according to the nature of the substrate and the age of the colony.

Shapes of Cells.—The individual yeast cells are usually spherical, ovoid, or ellipsoid in form. Broadly egg-shaped and elongated sausage-shaped cells may be produced characteristically by certain yeasts,

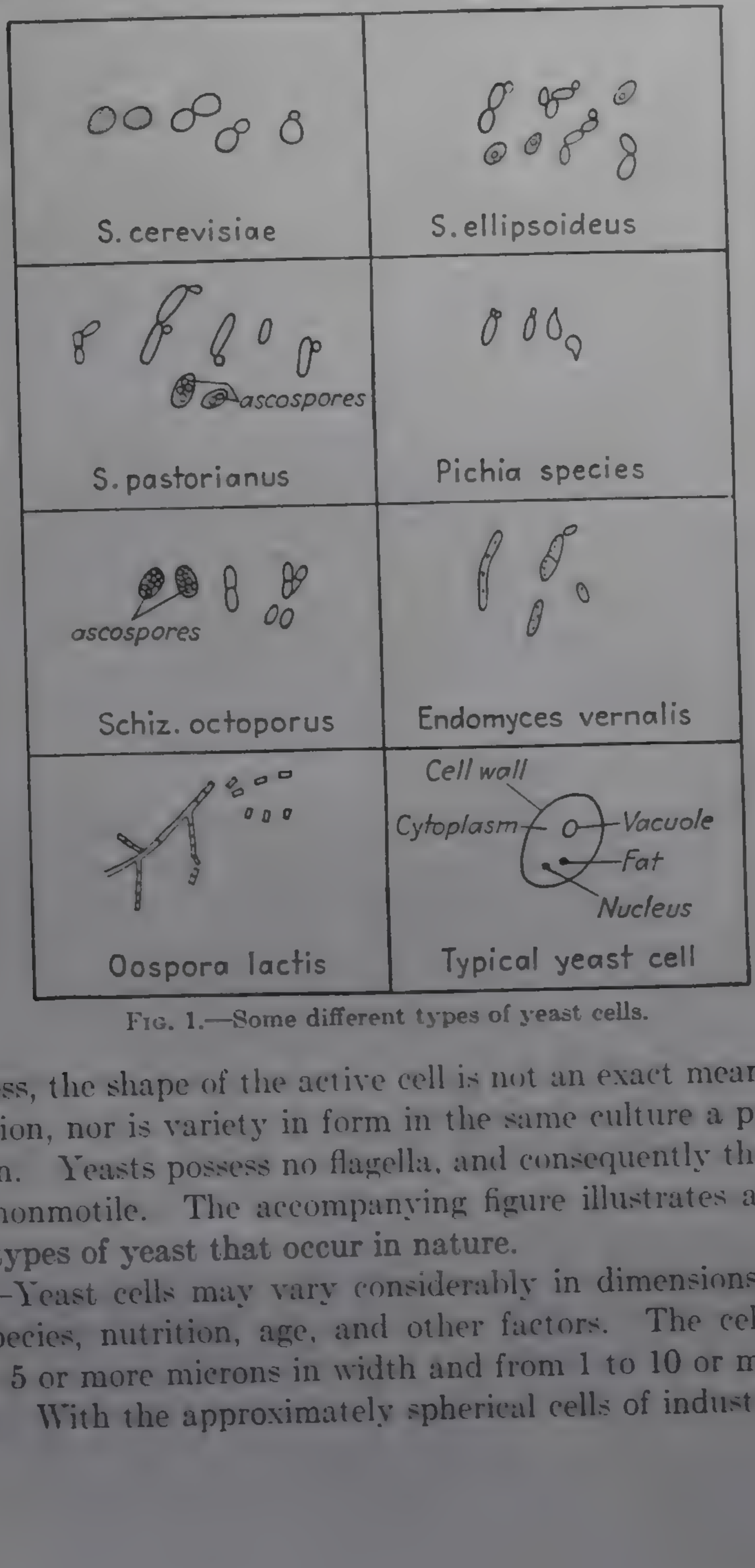


FIG. 1.—Some different types of yeast cells.

nevertheless, the shape of the active cell is not an exact means of species identification, nor is variety in form in the same culture a proof of contamination. Yeasts possess no flagella, and consequently the individual cells are nonmotile. The accompanying figure illustrates a few of the different types of yeast that occur in nature.

Size.—Yeast cells may vary considerably in dimensions, depending on the species, nutrition, age, and other factors. The cell may vary from 1 to 5 or more microns in width and from 1 to 10 or more microns in length. With the approximately spherical cells of industrial yeasts a

diameter of 4 to 6 microns is probably a fair average, but great variations may occur even in the same culture. Most of the yeasts of extremely small size are of no present industrial importance except as they occur as contaminants.

In general, yeast cells are much larger than bacterial cells and could not be mistaken for the latter when observed microscopically. There are exceptions, however; some large bacteria exceed in size the smallest known yeasts mentioned in the figures given.

The Cell Wall.—A transparent permeable wall surrounds each cell or mass of protoplasmic material. The exact composition of the cell wall is not known. It is believed to be composed of "fungus cellulose," which differs in its chemical character from the cellulose complexes constituting the walls of green plants. The cell-wall membrane may be invisible or very thin in young cells but becomes thickened in old cells.

Contents of the Cell.—The protoplasm appears microscopically as composed of a grayish, finely granular, semifluid mass. Presumably it is of albuminous material intimately mixed with a "cell sap" of water with organic materials and salts in solution. Within the protoplast there is a nucleus and one or more rounded structures known as "vacuoles," which were originally so called because they appeared as clear or empty spaces but are now known to be the seat of reserve foods and finely divided "metachromatic granules" or volutin. The vacuoles are not conspicuous in very young cells but appear prominently in mature or old cells, and their nature can be somewhat determined by special staining methods. Generally a large vacuole is located near the nucleus, or there may be two or even more with a general polar location. The nucleus exists as a small mass near the center of the cell and cannot generally be seen without employing a special staining procedure. Iron hematoxylin is probably the best stain for this purpose. With this treatment the nucleus sometimes appears as a fairly compact body, but more often as a diffuse cluster of granules. The nucleus assumes an important role in reproduction. When budding takes place the nucleus moves toward the pole, and a portion of these granules (possibly half of them) migrates into the newly developing daughter cell.

Within the yeast cell protoplasm are numerous other substances or reserve materials in the form of droplets or granules, some of which are exceedingly complex in nature. In old cultures, some cells sometimes become thick walled and much enlarged and densely packed with these reserves. Such cells are sometimes called "durable" or "durative" cells, as they seem to have unusual resistance to adverse conditions.

These stored foods—including some carbohydrates (not starch), fats, or oils as refractile droplets, and some granules supposed to be proteins—

are normal constituents of all mature yeast cells but especially of the older cells, when nutritive conditions are favorable. As stated, this reserve food is found dissolved in the cell sap in vacuoles and in the form of granules or droplets.

The most important carbohydrate is glycogen $(C_6H_{10}O_5)_x$, but other polysaccharides have been detected. Trehalose, a disaccharide $(C_{12}H_{22}O_{11})$ yielding glucose when hydrolyzed by acids, phosphatides, enzymes, vitamins, growth substances, volutin, and, in some instances, pigments are found in yeasts.

Some yeasts store relatively large quantities of fat (see the section on fat production). One yeast, *Endomyces vernalis*, produces so much fat under suitable conditions that it was used as a source of fat for human consumption during World War I. Fat collects in vacuoles that often become highly refractile as the cell ages. One school of microbiologists regards the storage of fat as an indication of cell degradation.

Volutin, also referred to as metachromatic granules, occurs in vacuoles, one of which is nearly always located close to the nucleus. It is a reserve material and is believed to be nucleic acid or a nucleic acid compound. It is found usually in old cells, but tends to disappear during spore formation.[1]

Stains.—A list of some of the stains used in detecting the presence of various chemical substances in the yeast cell follows:

TABLE 2.—SOME STAINS USED TO DETECT SPECIAL SUBSTANCES IN THE YEAST CELL

Stain	Substance	Color produced in substance
Flemming's solution[1]	Fat globules	Brown to black
Sudan III	Fat globules	Red
α- or β-Naphthol plus dimethyl-*p*-phenylenediamine in a weakly alkaline sol	Fat globules	Blue
Dimethyl amido-azobenzene	Fat globules	Yellow
Neutral red (0.1%)	Metachromatic particles, vacuoles	Light pink to red
Polychrome methylene blue	Nucleoprotein of volutin	Blue
Zinc chloriodide	Cellulose	Blue
Iodine in potassium iodide	Glycogen	Red-brown
Iodine in potassium iodide	(Starch)	Blue-violet

[1] Flemming's solution: 15 parts of a 1 per cent aqueous solution of osmic acid, 1 part of crystallized acetic acid, 4 parts of a 2 per cent aqueous solution of chromic acid.

[1] HENRICI, A. T., "Molds, Yeasts and Actinomycetes," John Wiley & Sons, Inc., New York, 1930.

Chemical Composition.—Yeasts contain 68 to 83 per cent of moisture,[1] while bacteria contain 73.3 to 98.3 and molds 84.3 to 88.7 per cent. Protein, carbohydrate, fat, and ash contents vary, depending on the species of yeast and the conditions under which it was grown, the ash content of yeasts usually varying from 3.8 to 8.8 per cent on the basis of the dry matter. In the following table is an analysis of the dry matter, which constituted 30 per cent of the total weight of yeast.[2] It will be noticed that yeast is rich in protein and glycogen, also that phosphorus and potassium combinations make up about 91 per cent of the ash. Phosphates play a very important role in fermentation.

TABLE 3.—COMPOSITION OF THE DRY MATTER OF YEAST[1]

	Per Cent		Per Cent
Protein	52.41	Ammonia	8
		Purine and pyrimidine bases	12
		Diamino acids	20
		Monamino acids	60
Fat	1.72	Phosphorus pentoxide	54.5
Glycogen	30.25	Potassium oxide	36.5
Cellulose, gum, etc.	6.88	Magnesium oxide	5.2
Ash	8.74	Calcium oxide	1.4
		Silicon oxide	1.2
		Sodium oxide	0.7
		Sulphur trioxide	0.5
		Chlorine and iron	Trace
	100.00		

[1] FRED, C. N. History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.*, **22**: 1154 (1930).

The proteins of yeast include albumin, globulin, phosphoproteins, nucleoproteins, lecithoproteins and glycoproteins. Soluble protein derivatives—peptones, polypeptides, and amino acids—are normal constituents of the cell.

Reproduction.—The true yeasts, *Endomycetaceae*, may reproduce vegetatively by budding or by fission and also by spore formation.

During budding, cytoplasm and nuclear material flow from the mother cell into a growing bud or protruding portion of the cell (Fig. 2). The bud becomes constricted at its base, a wall forms across the channel, and the cell may break off at once or remain attached to the mother cell for a while. Not infrequently the mother cell may form buds in two or more places at once. Clusters of cells thus result when the new cells remain temporarily in connection with the parent cells.

[1] BUCHANAN, R. E., and E. I. FULMER, "Physiology and Biochemistry of Bacteria," Vol. I, Williams & Wilkins Company, Baltimore, 1928.

[2] FRED, C. N., History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.*, **22**, 1154 (1930).

FIG. 2.—Budding yeast cells. Magnified 1,500 times. Pictures taken at 15-minute intervals. (*Courtesy of Dr. C. N. Frey of the Fleischmann Laboratories.*)

Another vegetative type of reproduction is transverse fission. This process is similar to that of bacterial fission. The mother cell grows, elongates, becomes somewhat constricted in the middle, and a division wall is formed, two new cells resulting. The genera *Schizosaccharomyces* and *Endomyces* reproduce vegetatively only by transverse fission.

Sporulation, a reproductive process in true yeasts, may be of asexual

or sexual nature. The spores, of which there may be 1 to 4, 6, 8, or very infrequently 12, are known as "ascospores" or "endospores," and the cells that contain the spores are known as "ascs" or "asci" (singular, "ascus").

True yeasts are classified as *Ascomycetes* (sac fungi).

In ordinary ascospore formation, the nucleus of the yeast cell divides repeatedly. Each nucleus is then surrounded by dense cytoplasmic material and finally a wall.

When the nuclei of two different yeast cells fuse prior to the division of the nucleus, the resultant spores are sexually produced in nature—this type of spore formation is much less common than the asexual method, *i.e.*, without fusion.

Conjugation may be isogamic or heterogamic. In isogamic conjugation the nuclei of two neighboring cells of about equal magnitude fuse. In heterogamic conjugation, the nucleus of the bud fuses with the nucleus of the mother cell, or nuclei of cells of very unequal dimensions fuse. The fusion nucleus divides and the ascospores form. The genera *Debaryomyces* and *Zygosaccharomyces* illustrate isogamic and heterogamic conjugation.

Yeast Genetics.—The nucleus of the yeast cell is concerned with heredity. Each nucleus contains one or two sets of *chromosomes*, the bearers of heredity. The number of chromosomes in a basic set is usually constant for a species. For example, the fruit fly (*Drosophila melanogaster*) contains four chromosomes in its basic set, while man (*Homo sapiens*) contains 24 chromosomes in the basic set. Each chromosome in turn contains *genes*, the determiners of heredity.

A reproductive cell is known as a "gamete," which in the higher forms of life is usually designated as an egg or sperm. However, in the lower forms of life, where no simple method is available for differentiating between the cells, they may be referred to as male and female or as plus or minus cells. Each gamete carries one set of chromosomes in its nucleus. The basic number of chromosomes for a species is known as the "haploid" or "monoploid" number. This may be designated as the $1n$ or $1x$ number. Twice the basic number of chromosomes, such as is found in a zygote resulting from the fusion of two gametes, is known as the "diploid" number and may be designated as the $2n$ or $2x$ number.

When two gametes of the same sex (or mating types of the same kind) fuse, there results a homozygous diploid, which is known as an "illegitimate" diploid. Thus, the union of two plus or two minus gametes of haploid nature gives rise to an illegitimate diploid. On the other hand, the fusion of a plus gamete with a minus gamete gives rise to a heterozygous diploid, known as a "legitimate" diploid.

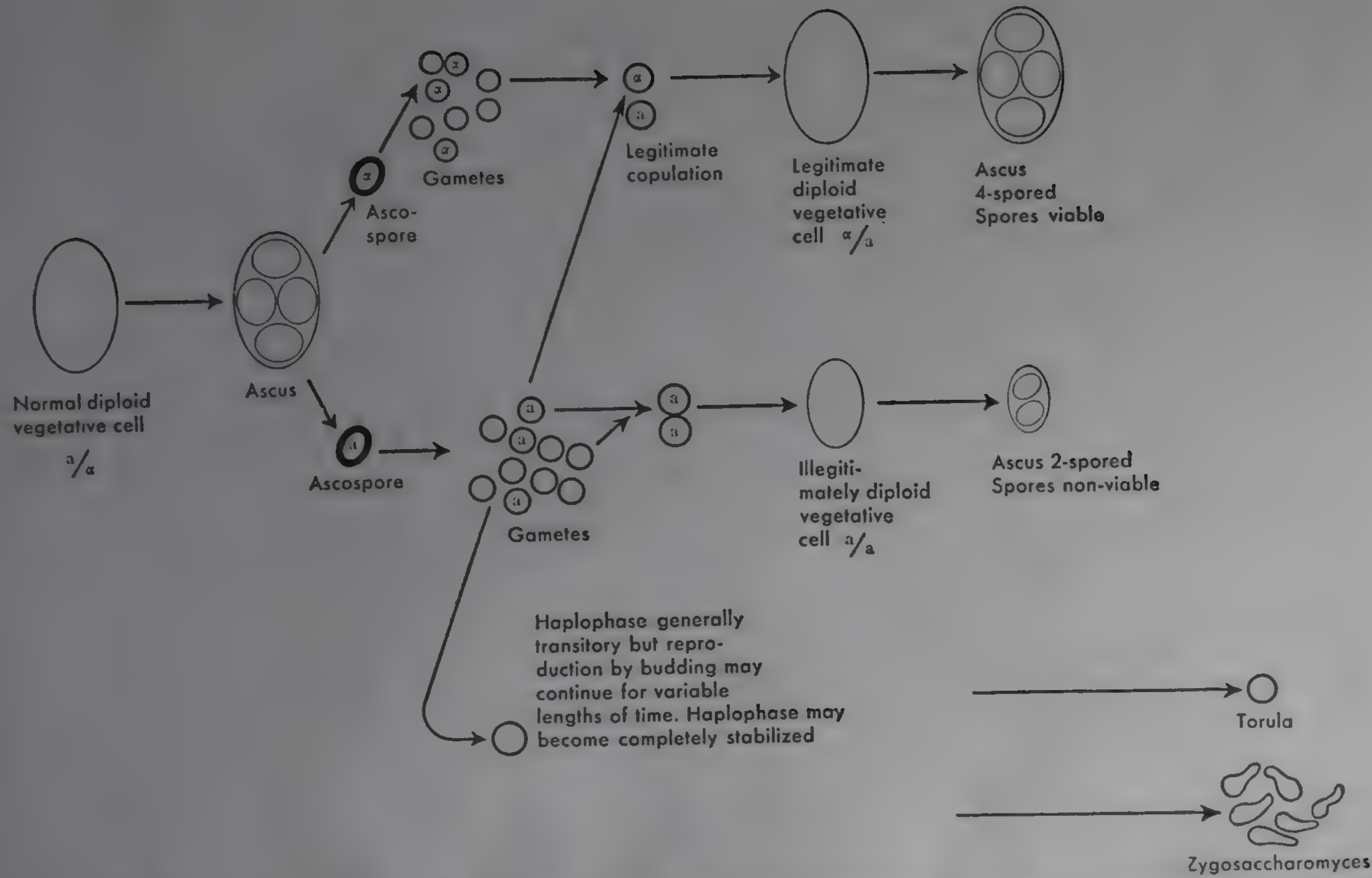


FIG. 3.—Life cycle of *Saccharomyces cerevisiae* showing the relationship with *Torula* and *Zygosaccharomyces*. The ascospores belong to two mating types *a* and α, and the heterozygote is the legitimate diploid. The homozygotes are called illegitimate diploids. [*Courtesy of C. C. Lindegren, Wallerstein Labs. Communs.*, **7** (No. 22):153(1944).]

myces validus. The resultant hybrid was an improvement over the standard bakers' yeast. A hybrid produced by mating haplophase cultures of *S. cerevisiae* with haplophase cultures of *S. carlsbergensis* was able to synthesize biotin, pantothenic acid, and pyridoxine, vitamins of the B-complex, in large amounts, according to Lindegren and Lindegren.[1,2] *S. cerevisiae* was unable to synthesize either biotin or pantothenic acid but able to synthesize pyridoxine. *S. carlsbergensis* was able to synthesize both biotin and pantothenic acid in large amounts but unable to synthesize pyridoxine.

The subject of genetics, as related to yeasts, is a large and growing one. The interested reader is referred to the end of this chapter for a list of references. The papers by the Lindegrens and Spiegelman in this country and by Winge and Laustsen of the Carlsberg Laboratory, Copenhagen, are particularly significant.

Yeast Spores.—Sporulation in yeasts is important for two reasons: it is the basis for a method of reproduction, and it serves an important role in maintaining the viability of yeasts during adverse changes in the environment. The ascospores of yeasts are more resistant to heat and desiccation than the vegetative cells, yet they are much less resistant to heat than bacterial spores.

Sporulation.—Sporulation may be initiated by a deficiency in the food supply and the accumulation of toxic end products[3] but will not proceed unless certain other conditions are favorable, namely, the yeast cells must be young and vigorous; there must be plenty of air and moisture available; the pH of the medium must be suitable; inhibitory substances must be absent; and the temperature of incubation must be satisfactory. The presence of stimulating substances promotes sporulation.

In order to produce vigorous young cells, the yeast should be cultured in a suitable medium and transferred frequently. Reserve foods—glycogen, fat, and other products—are stored in the yeast. The use of such cells will insure sporulation, provided the other conditions are satisfactory and the yeast has the ability to form endospores.

Oxygen is essential for sporulation; without it no spores are formed.

Temperature is important. Each variety of yeast sporulates most readily in a given temperature range. Above certain temperatures and below others, sporulation fails to take place. Table 4 shows the maximum, minimum, and optimum temperatures for the sporulation of six varieties of yeasts studied by Hansen.

[1] LINDEGREN, C. C., and G. LINDEGREN, *Science*, **102** (No. 2637): 33–34 (1945).
[2] LINDEGREN, C. C., *Missouri Botan. Garden Bull.* **34**: 37–43 (1946).
[3] GUILLIERMOND, A., "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

Acc No: 13013

FIG. 4.—Sporulation of yeast. (*Courtesy of H. Kothe and F. R. Swift, The Fleischmann Laboratories.*)

The optimum temperatures for the six varieties lie between 25 and 30°C., while three varieties have an optimum temperature of 25°C. At the most favorable temperature for sporulation, ascospores begin to appear in 21 hr. or more.

Calcium sulphate stimulates sporulation but restrains budding. Beer

TABLE 4.—MAXIMUM, MINIMUM, AND OPTIMUM TEMPERATURES FOR SPORULATION OF CERTAIN YEASTS[1]

Yeast	Maximum temperature, degrees Centigrade	Minimum temperature, degrees Centigrade	Optimum temperature, degrees Centigrade
Saccharomyces cerevisiae	35 –37	9 –11	30
Saccharomyces pastorianus	29 –31.5	0.5– 4	27.5
Saccharomyces intermedius	27 –29	0.5– 4	25
Saccharomyces validus	27 –29	4.8– 5	25
Saccharomyces ellipsoideus	30.5–32.5	4.7– 5	25
Saccharomyces turbidans	33 –35	4 – 8	29

[1] Reprinted by permission from Guilliermond, "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

containing glucose or other sugars or gelatin stimulates sporulation, but ammonium salts have an adverse affect. Green, blue, violet, and ultraviolet rays have inhibitory effects on the formation of endospores. Extremes of pH definitely retard sporulation.

Methods of Inducing Sporulation.—There are a number of methods for inducing sporulation, some of which have been reviewed by Henrici[1] and by Lindegren and Lindegren.[2] They involve the use of nonnutrient, vegetable and/or fruit, and other types of substrates. Failure of a medium to induce sporulation in an unknown yeast does not necessarily mean that the yeast is asporogenous. Before arriving at such a conclusion, it may be necessary to try several different methods.

1. *Plaster of Paris Block.*—In this method, introduced by Engel, the upper surface of a block molded from pure plaster of Paris is scraped smooth. The block is placed in a glass container, which is fitted with a cover, and water is added until the block is about one-half submerged. The container, cover, and contents are then sterilized. Yeasts cells from a young, vigorous culture, which has been grown on a suitable medium with frequent transfer, are placed on the smooth surface of the block, and the container with contents is incubated at the desired temperature (25 to 30°C. usually) for 30 hr. or longer before observations are made. Hansen has devised a special flask for use in this method. Clay and blotting paper have been substituted for plaster of Paris.

2. *Plaster of Paris Slants.*—This is a modification and improvement made by Graham and Hastings.[3] Plaster of Paris is mixed with an equal weight of water and introduced into bacterial culture tubes in appropriate amounts (about 10 ml.). The tubes are placed in a slanted position in a drying oven at a temperature of approximately 50°C. and allowed to dry. Afterward the slants are plugged with cotton and sterilized.

Lindegren and Lindegren, who used such slants in combination with their presporulation medium (to be described later), advocated the addition of 3 ml. of sterile water, acidified to a pH of 4 with acetic acid, to the base of each slant at the time of its use.

3. *Carrot Infusion Agar* (*McKelvey*).—Finely subdivided unpeeled carrots are autoclaved with water. The solids are separated from the extract by filtration and light pressure. Sufficient agar is added to the extract to solidify it (about 20 g. per liter). Before adding the carrot infusion agar to the culture tubes, a small amount of calcium sulphate is placed in each. The medium is sterilized and slanted.

[1] Henrici, A. T., *Bact. Revs.*, **5**: 97–179 (1941).
[2] Lindegren, C. C., and G. Lindegren, *Botan. Gaz.*, **105** (No. 3): 304-316 (1944).
[3] Graham, V. R., and E. G. Hastings, *Can. Jour. Res.*, **19**: 251-256 (1941).

4. *Sporulation Stock Medium.*—Mrak, Phaff, and Douglas[1] have devised a medium which has produced good sporulation within 1 week or less with several hundred yeast cultures and which serves also as an excellent stock-culture medium. This medium is prepared by grinding equal weights of washed and unpeeled beets, carrots, cucumbers, and potatoes, mixing with a weight of water equal to the combined weights of the vegetables used, autoclaving the mixture for 10 min. at a pressure of 10 lb. per sq. in., separating the solids from the extract by filtering through cheesecloth and applying some pressure, adding 2 per cent of agar to the extract, distributing the infusion agar into culture tubes, sterilizing for 15 min. at a steam pressure of 15 lb., and slanting. The pH of the extract is reported to be about 5.7.

5. *Presporulation Medium.*—Lindegren and Lindegren[2] have reported the achievement of good results through the combined use of a presporulation medium and plaster of Paris slants, although the presporulation medium itself will produce endospores if incubated for a few weeks. The presporulation medium devised by Lindegren and Lindegren contains the following ingredients:

Beet leaves extract	10 ml.
Beet roots extract	20 ml.
Apricot juice	35 ml.
Grape juice	16.5 ml.
Yeast dried	2 g.
Glycerol	2.5 ml.
Calcium carbonate	1 g.
Agar	3 g.
Water to	100 ml.

The medium is steamed for 10 min. and then distributed into culture tubes. The tubes are sterilized for 20 min. at 15 lb. and cooled in a slanted position. If endospores are desired within a short time, the procedure is as follows: Approximately 1 ml. of sterile water is pipetted or poured over the surface of a 3-day growth of the yeast on the presporulation medium. The tube is allowed to stand for 10 min. and then a suspension of the yeast cells is made in the water. This suspension is pipetted to the upper portion of a plaster of Paris slant. About 3 ml. of sterile water, acidified to a pH of 4 with acetic acid, are pipetted onto the bottom of the slant. The inoculated slants are incubated at 25°C. for 1 to 2 days and then examined for endospores.

[1] Mrak, E. M., H. V. Phaff, and H. C. Douglas, *Science*, **96** (No. 2497): 432 (1942).

[2] Lindegren and Lindegren, *loc. cit.*

6. *Vegetable Juice Medium.*—A medium prepared from a commercially available canned blend of eight vegetable juices with sufficient agar to solidify it has been used by Henrici[1] to obtain sporulation of yeasts.

Wickerham, Flickinger, and Burton[2] have modified Henrici's medium and reported a "high degree of sporulation." The blend of eight vegetable juices from a can containing 1 pt. and 2 fl. oz. is adjusted to a pH of 6.8 with potassium hydroxide. One-half of a cake of compressed yeast is suspended in the juice and steamed for 10 min. to destroy the yeast cells and to liberate some of their contents.

The medium is again adjusted to pH 6.8 and then added to an equal volume of hot distilled water containing 4 per cent of melted agar. After mixing, the medium is dispensed into tubes or bottles and sterilized for 15 min. at a steam pressure of 15 lb.

Not sooner than 8 hr. before use, the medium is freshly slanted. The whole surface of the slant is inoculated lightly with a 24-hr. culture of the yeast, which has been grown on a slant of yeast extract malt extract agar at about 28°C. According to Wickerham and his associates, good sporulation of species of *Hansenula*, *Zygohansenula*, *Pichia*, and *Zygopichia* may be expected to take place within 3 days, of *Saccharomyces* and *Zygosaccharomyces* within 5 to 7 days, and of *Debaryomyces* within 5 to 20 days.

The junior author suggests that canned vegetable mixtures could also be used in the preparation of a sporulation medium. The vegetables could be used instead of fresh ones for the preparation of an infusion. The filtered extract would be solidified with agar.

7. *Other Vegetable and Fruit Substrates.*—Carrot plugs, potato plugs, cucumber wedges, grape juice, prune juice, raisins, cherry juice, raisin agar, and other substrates have been used successfully to induce sporulation of yeasts.

8. *Gorodkowa's Medium.*—Satisfactory results have been obtained by inoculating slants containing Gorodkowa's medium with an active culture of yeast:

Glucose	0.25 g.
Beef extract	1.00 g.
Agar or gelatin	1.00 g.
Sodium chloride	0.50 g.
Distilled water	100.00 ml.

9. *Kufferath's Medium.*—Malt is hydrolyzed with sulphuric acid and neutralized to various pH levels with calcium carbonate and sodium hydroxide. Agar is added, and the media are sterilized and inoculated.

[1] Henrici, *loc. cit.*

[2] Wickerham, L. J., M. H. Flickinger, and K. A. Burton, *Jour. Bact.*, **52** (No. 5): 611–612 (1946).

10. *Soil Extract Gelatin Medium.*—This medium, developed by Niehaus (1932), is prepared by extracting 1,000 to 1,500 g. of garden soil with 1,000 ml. of water, by separating the solids from the extract by filtration, and by adding 15 per cent gelatin.

11. *Other Methods.*—Various other methods have been used for inducing yeasts to sporulate. Sterilized sausages have been used by Stelling-Dekker for sporulation of *Debaryomyces* species. Endospore formation may be occasionally induced in compressed yeast by storing it for 6 to 8 days in a refrigerator. Beechwood chips, soaked and sterilized, have been used by at least one investigator. Sugar solutions in shallow layers have been employed successfully.

The Isolation of a Single Spore.—Winge and Laustsen[1] regard the one-spore culture as being the smallest biological unit in yeasts instead of the single cell.

FIG. 5. Chamber used in isolating single spores. (*Courtesy of Dr. Ö. Winge, Carlsberg Laboratory, Copenhagen.*)

Laustsen has developed a technique for isolating all the spores of an ascus and cultivating them. His method is as follows:[1] The yeast is caused to sporulate by placing it on a plaster block and incubating it for at least 30 hr. at room temperature. Using aseptic technique, a small drop of wort on a cover slip is inoculated with the spore material. The cover slip is then placed on a special operating chamber (Fig. 5).

An ascus is selected and withdrawn from the droplet, using a very fine glass needle. A small amount of wort naturally adheres to the ascus—more may be readily added if desired.

The isolation (Fig. 6) is carried out by means of two special glass needles. One needle, which has a point about 7 microns in diameter, is

[1] WINGE, Ö., and O. LAUSTSEN, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22** (No. 6): 99 (1937).

introduced through one opening of the operating chamber (Fig. 5); the other needle, which has a point approximately 2.5 microns in diameter, is introduced through the other opening. The two needles are manipulated in such a manner as to burst the wall of the ascus against the surface of the cover slip. Each of the spores liberated is pulled into a separate droplet of sterile wort, which has been placed previously on the cover slip.

After the spore has germinated and a colony of cells has developed, some of the cells are transferred by the use of a sterile platinum needle to a Freudenreich flask containing sterile wort. Some of the remaining

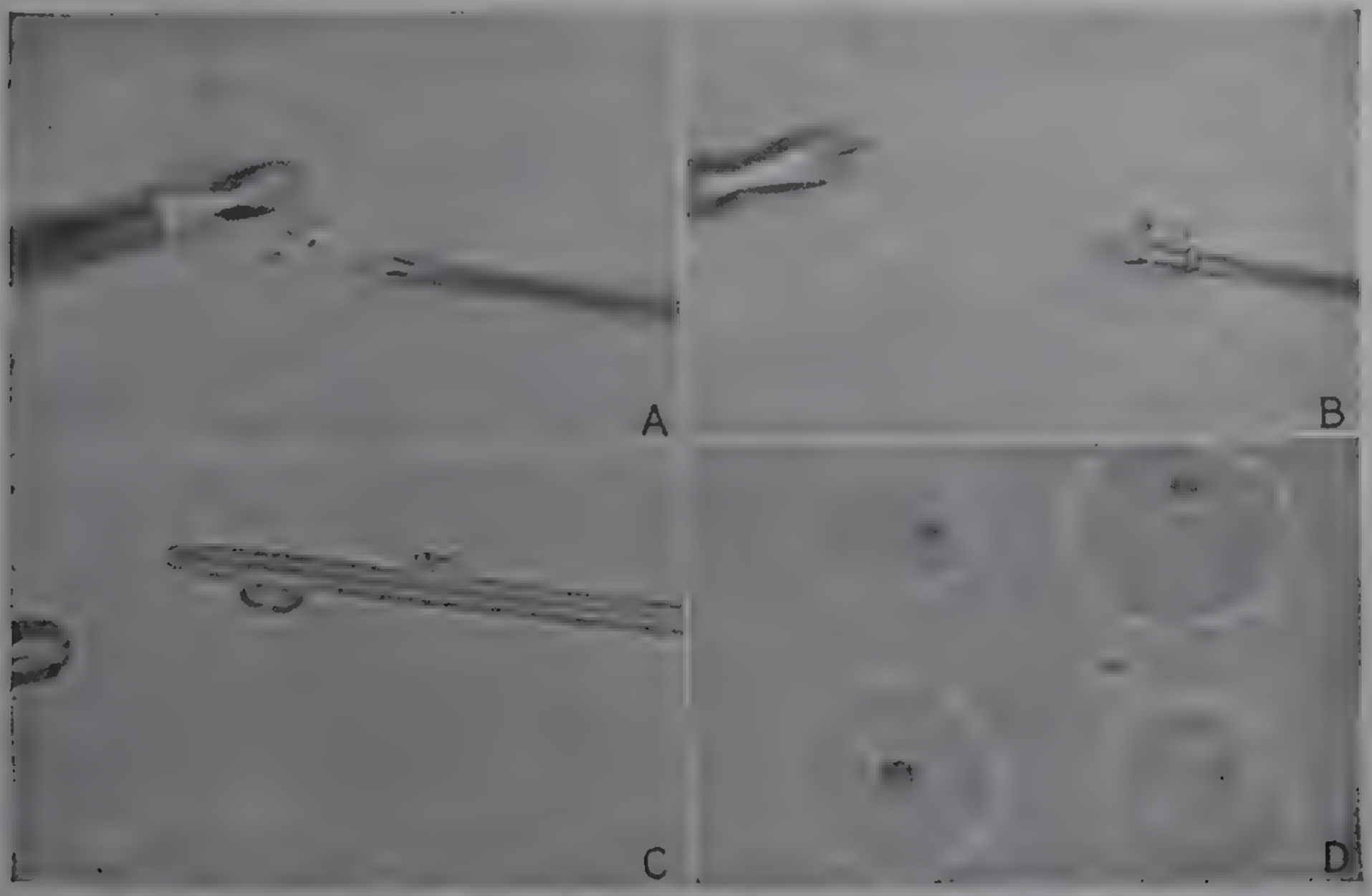


FIG. 6.—The isolation of single spores. (*Courtesy of Dr. Ö. Winge, Carlsberg Laboratory, Copenhagen.*)

cells may be transferred to a second flask by using a sterile, pointed piece of filter paper.

Occurrence and Distribution of Yeasts.—The budding fungi are of wide distribution in nature. Their habitats may include not only the upper layers of the soil but also many forms of organic matter, especially of plant origin, where carbohydrates are of common occurrence. Yeasts may be isolated particularly from the soil of vineyards and orchards; from the surfaces of grapes, apples, and most sweet fruits; from citrus fruits; and from the leaves and other parts of plants. They are carried into the air with dust and on the bodies of insects and thus may be widely disseminated. Proctor[1] has shown that yeasts may be found in the air at high altitudes. Some types of yeasts are also found occasionally on animal products.

[1] PROCTOR, B. E., *Proc. Am. Acad. Arts Sci.*, **69**: 315 (1934).

Methods of Isolating Pure Cultures of Yeast.—In order to make sure that the culture isolated is pure and that it is not in reality a mixed culture of two morphologically related varieties of yeast or an undesirable combination of microorganisms, it is necessary to isolate single cells by special technique and to observe these cells during reproduction. Although there are several methods by which pure cultures may be isolated, the methods cited here will be restricted to the more important ones. Skill and patience are required in some of the methods of single-cell isolation.

1. *Moist-chamber Method of Hansen.*[1]—The chamber (Fig. 7) consists of a glass slide, a glass ring, and a cover slip with numbered squares (usually 16). The cover slip is attached to the ring by glass cement,

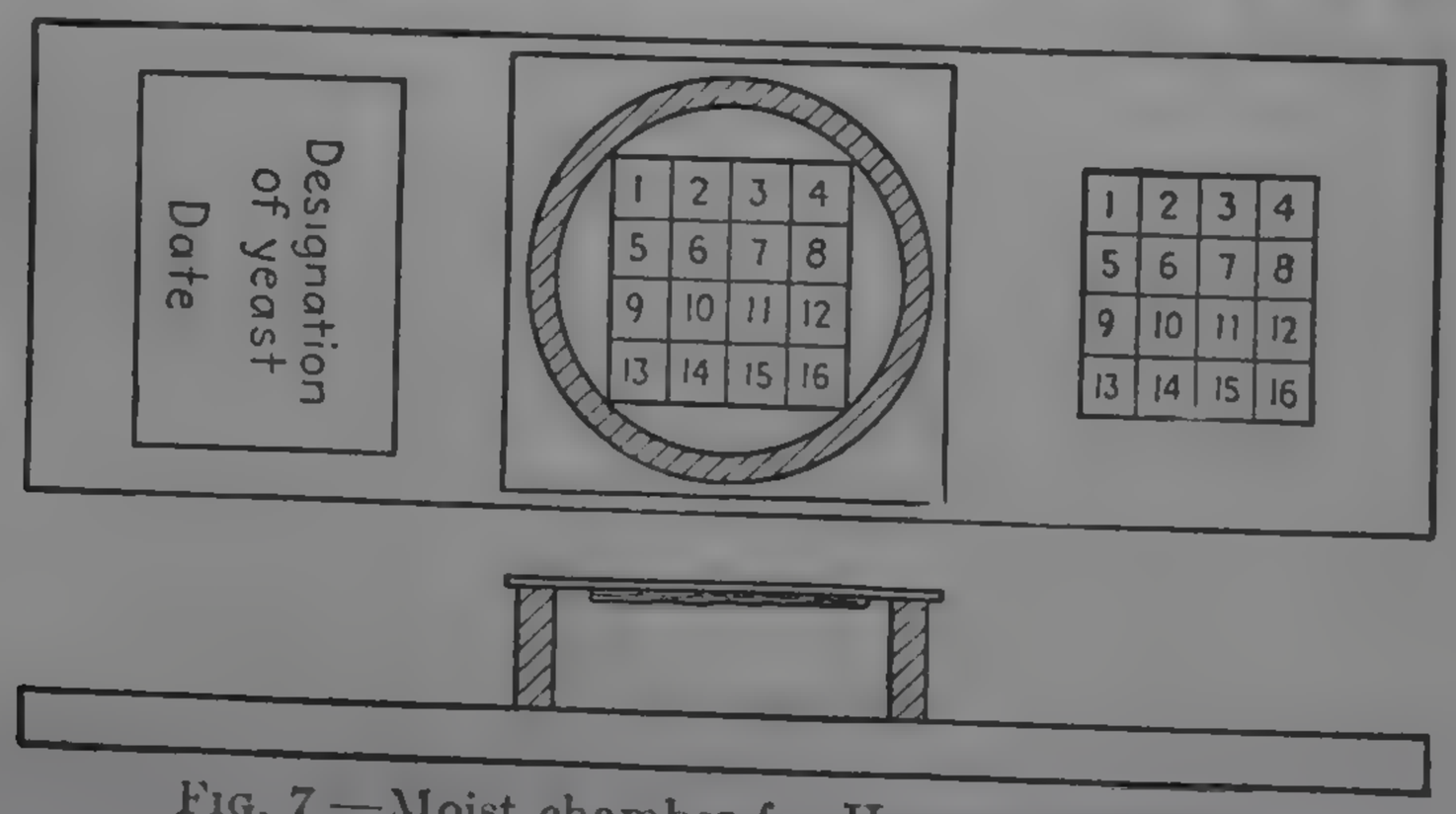


FIG. 7.—Moist chamber for Hansen method.

wax, or a vaseline-wax mixture. Before using the chamber, the component parts should be sterilized by flaming them carefully.

The culture of yeast from which the single-cell isolation is desired is diluted with sterile beer wort or water until a drop contains only a few cells. A drop of the diluted culture is then placed in a tube of sterile melted wort gelatin, and the tube is thoroughly agitated to distribute the yeast cells uniformly throughout the medium. One drop of this medium is spread out on a glass slide, and, using the 100 × magnification of a microscope, the slide is examined for the approximate number of cells. If the number is satisfactory (20 or less), a small drop of the liquid wort gelatin containing the cells is placed on the underside of the cover slip and spread thinly and uniformly over the numbered squares. The chamber is placed so that the gelatin rests in an even layer on the cover slip until it has solidified. Afterward the chamber is inverted, a drop of sterile water is placed on the slide to ensure a moist atmosphere in the chamber, and the ring is sealed tightly to the slide by means of vaseline.

[1] JORGENSEN, A., "Practical Management of Pure Yeast," revised by A. Hansen, Charles Griffin & Company, Ltd., London, 1936.

The single cells are then located microscopically, and their positions are mapped on a diagram corresponding to the marked cover slip. Several moist chambers are usually prepared at the same time. They are then incubated at room temperature or around 25°C. for 2 or more days, during which time the growing yeast cells are carefully observed. During this time the single cells will have developed into colonies. Tubes of sterile wort are then inoculated from the individual colonies known to have grown from single cells.

2. *The Lindner Method.*—This method is a modification of the Hansen procedure. The culture containing the desired yeast is diluted in sterile wort, cider, or grape juice. Using a sterile crow-quill pen or wire, five rows of droplets, 5 droplets to a row, are deposited on a sterile cover slip that may or may not be marked into numbered squares. The cover slip is placed, culture side down, over the concavity of a sterile hollow ground-glass slide or ring slide after placing a drop of sterile water in the chamber. It is sealed with vaseline or paraffin. The droplets are examined microscopically, and those containing a single cell are marked by drawing small circles around them, or, if the squares are numbered, the locations are recorded by sketching the position of the droplet on a corresponding drawing. After incubation and observation, the cultures are transferred to sterile wort.

3. *Dilution-plate Method.*—Tubes of dextrose, malt, or other suitable agar are melted and cooled to 42 to 44°C. Using a loop, a tube of agar is inoculated with the yeast-containing culture. The tube is thoroughly shaken, a unit amount of this medium is transferred to a second tube of melted agar. A third tube is inoculated in the same manner from the second tube. The contents of each of the three tubes are poured into petri dishes, and the plates incubated at 25 to 30°C. for 2 or more days. When the colonies have developed, samples of those which appear to consist of pure yeasts are examined with the microscope, using hanging drops. If the yeast appears to be pure and satisfactory, sterile wort may be inoculated from the colony. This method, though simple, does not ensure the isolation of a culture from a single cell.

4. *The Micromanipulator Method.*—The micromanipulator may be used successfully in isolating pure cultures of yeasts from single cells. In experienced hands, this method is rapid. The euscope, attached to the microscope, aids in the isolation of single cells.

Giant Colonies.[1]—Lindner suggested that giant colonies may contribute information that would be useful in identifying a yeast. A large

[1] GUILLIERMOND, A., "Clef dichotomique pour la détermination des levures," Librairie Le François, Paris, 1928; "The Yeasts," translated and revised by F. W. Tanner, *op. cit.*

horizontal surface of a suitable nutrient agar or gelatin medium contained in an Erlenmeyer flask or a large bottle is inoculated at one point in the middle with the aid of an inoculating needle. Petri dishes may be used, but owing to their susceptibility to contamination, they must be wrapped with surgeon's tape or packed in sealed containers to prevent contamination. Incubation is usually made for a period of 1 to 2 months at or near 20° C. The use of moist chambers aids in preventing the agar from shrinking prematurely.

Identification of Yeasts.—Yeasts are identified on the basis of a large number of observations—morphological, physiological, biochemical, cultural, and others. The shape and size of the cell; the optimum, minimum, and maximum temperatures for budding, sporulation, and film formation; the copulation of cells; the morphological nature of the asci and ascospores; the method in which ascospores germinate; the characteristics of the sediment formed in wort; the appearance of colonies grown on various solid media; the characteristics of giant colonies; and the biochemical characteristics, such as the action on various sugars, are some of the more important factors considered in identifying an unknown yeast. For an intensive review of this subject the reader is referred to the works of Hansen, Lindner, Guilliermond, Tanner, Kufferath, and others.

The Nutrition of Yeasts.—Yeasts, like bacteria and other forms of life, require certain food materials and environmental conditions for proper growth and reproduction. Some elements are basically necessary as, for example, carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium, sulphur, calcium, iron, and magnesium. There is accumulating evidence which indicates that trace elements also play an important role in nutrition. Vitamins and or other organic compounds are required for the satisfactory development or functioning of most types of yeasts. An adequate supply of water is essential for carrying out their life activities.

Carbon.—In considering sugars as carbon sources, one must be reminded that the ability of a yeast to assimilate a sugar may be quite different from its ability to ferment the same sugar. Likewise the ability to assimilate a given compound varies with different varieties of yeasts. Carbon may be supplied in the form of sugars, aldehydes, salts of some organic acids, glycerol, or ethanol.

Guilliermond has stated that impure maltose is best suited to the metabolism of yeasts.[1] Sucrose, glucose, fructose, and raffinose are not so important from the viewpoint of assimilation, yet some of these sugars are

[1] GUILLIERMOND, A., "The Yeasts," translated by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

readily fermented. Lactose is assimilated only in certain isolated cases.

Acetates, citrates, lactates, malates, succinates, and tartrates, as well as lactic, malic, succinic, and tartaric acids, may be used as sources of carbon.

Ethyl alcohol, in low concentration, occasionally may be utilized.

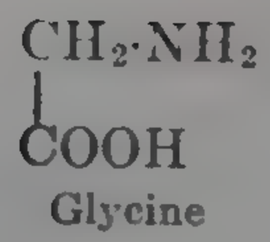
Nitrogen.—This element may be supplied to yeasts, depending upon the species or strain, in the form of ammonia, ammonium salts, amino acids, peptides, peptones (or other soluble protein derivative), nitrates, or urea. However, the last two are not usually very satisfactory sources of nitrogen for most yeasts.

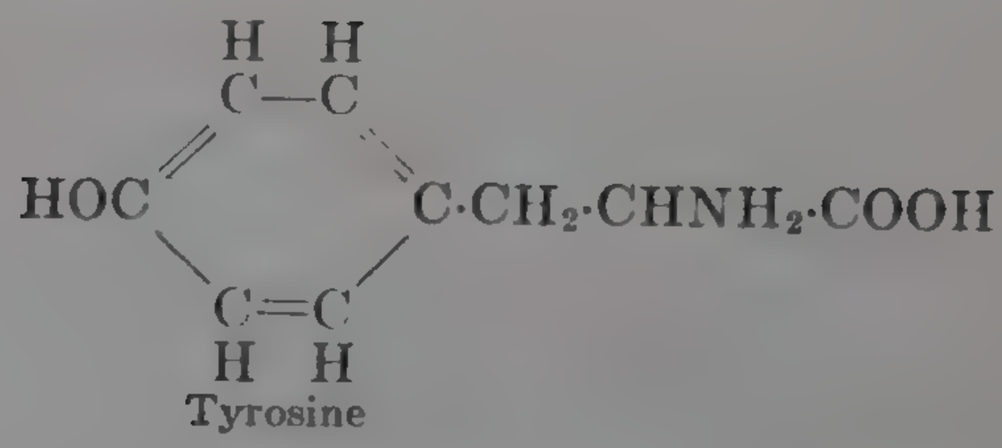
Ammonia and ammonium salts, particularly ammonium sulphate, appear to be most suitable sources of nitrogen on account of their availability, low cost, and ready assimilation. Ammonia is apparently preferred by yeasts to other sources of nitrogen when these are present in a medium. Amino acids are deaminized and the ammonia used by the yeasts.

Wort, which contains amino acids and other soluble nitrogen compounds (produced by the action of the proteolytic enzymes of malt on the proteins of barley), is an adequate and good source of nitrogen (refer to the chapter on Brewing).

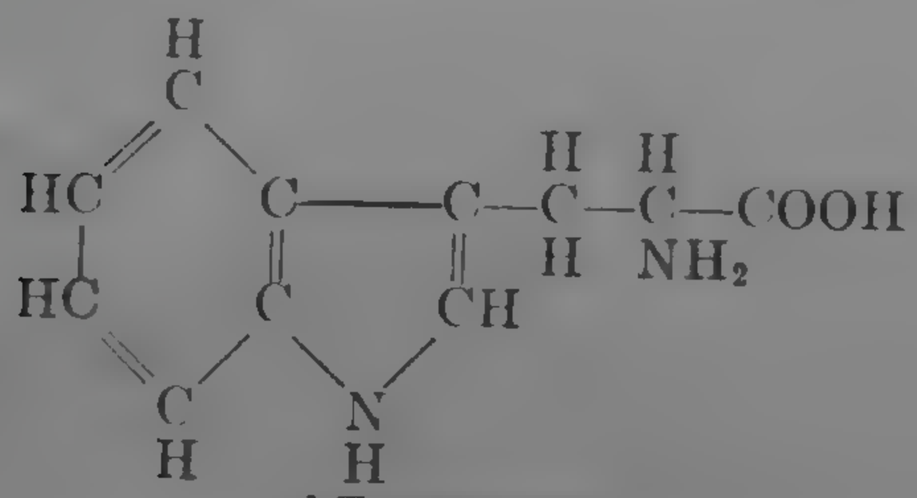
UTILIZATION OF AMINO ACIDS.—There is considerable difference among the amino acids in respect to their availability and value as nitrogen sources for yeasts. The difference depends in part upon the position of the amino group present and the isomeric form available. In most cases where the amino group is attached to the carbon atom adjacent to the carboxyl group (alpha position), the nitrogen from the amino group is utilized. For example, aspartic acid, glutamic acid, leucine, asparagine, glycine, and tyrosine are very good sources of nitrogen for yeasts. According to Nielsen,[1] 99 per cent of the nitrogen from aspartic acid, asparagine, and glycine is assimilated by yeasts. The structural formulas of these compounds follow:

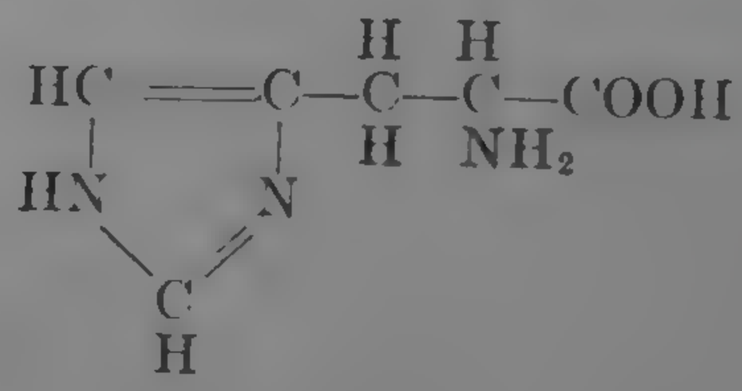
[1] NIELSEN, N., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **21**: 395 (1936); *Sér. chim.*, **22**: 384 (1937).


Glycine


Tyrosine

In some instances only a fraction of the total nitrogen is utilized. Only about one-half (56 per cent) of the nitrogen of tryptophane and one-third (34 per cent) of the nitrogen of histidine are used, indicating that the alpha-amino group is attacked.[1]


l-Tryptophane

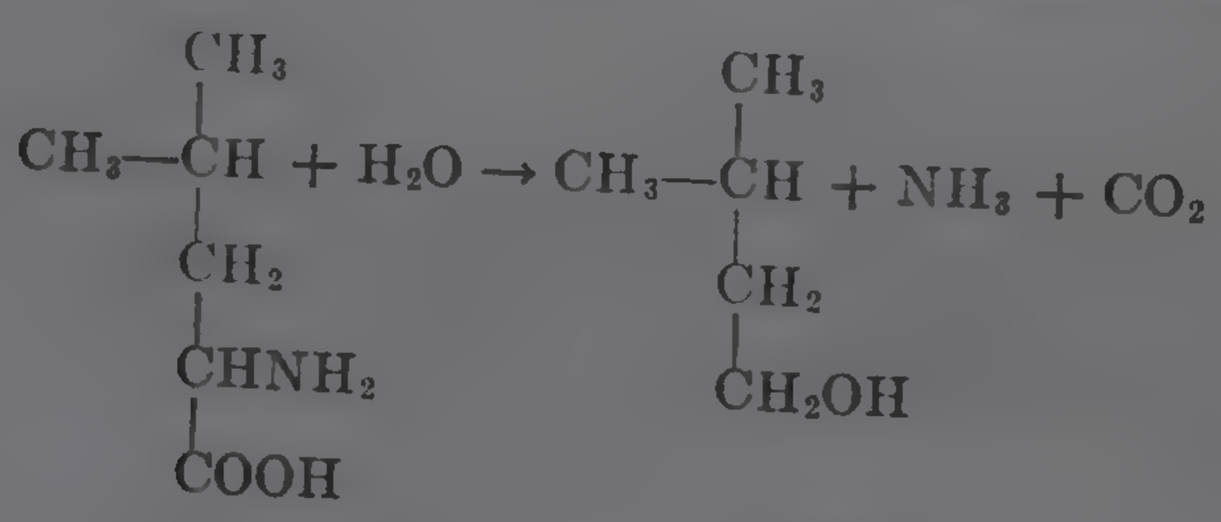

l-Histidine

All amino acids except glycine exist in two isomeric forms (the dextro and levo forms).[1] Yeasts appear to be able to utilize the form of amino acid that occurs naturally in preference to the synthetic isomer; but will utilize both isomers of some amino acids, for example, the dextro and levo forms of aspartic and glutamic acids and asparagine.

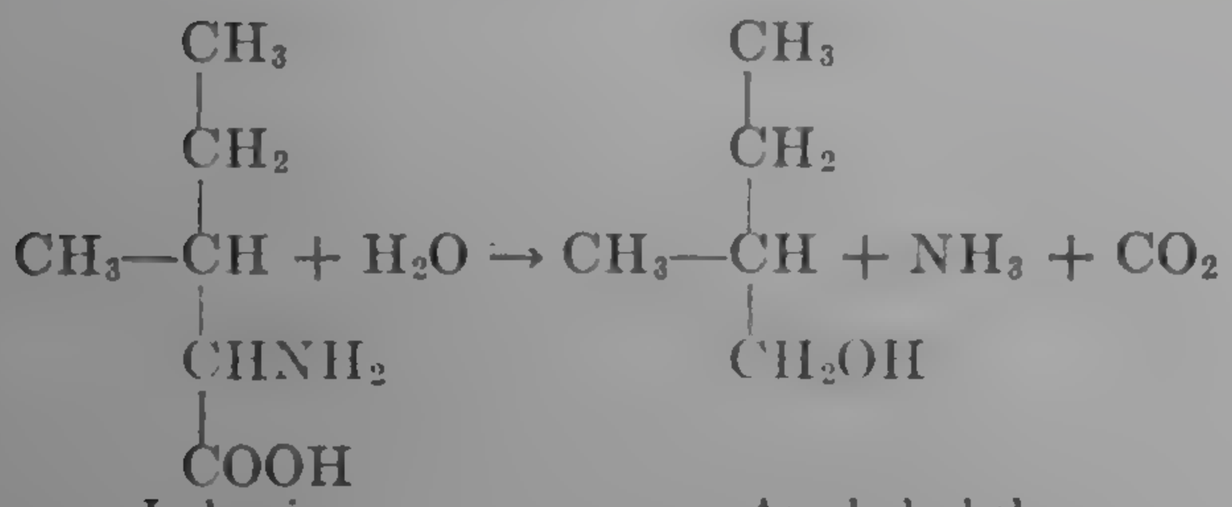
CONVERSION OF AMINO ACIDS TO ALCOHOLS.—Ehrlich has shown that certain amino acids, namely, leucine, isoleucine, phenylalanine, tryptophane, and tyrosine, are converted to alcohols by yeasts. His work has been confirmed by Thorne. The reaction is a hydrolytic deamination and decarboxylation. The amino group is hydrolyzed and the carboxyl group is decarboxylated in accordance with the following general reaction:

$$\underset{\text{Amino acid}}{R\cdot CHNH_2\cdot COOH} + H_2O \rightarrow \underset{\text{Alcohol}}{R\cdot CH_2OH} + NH_3 + CO_2$$

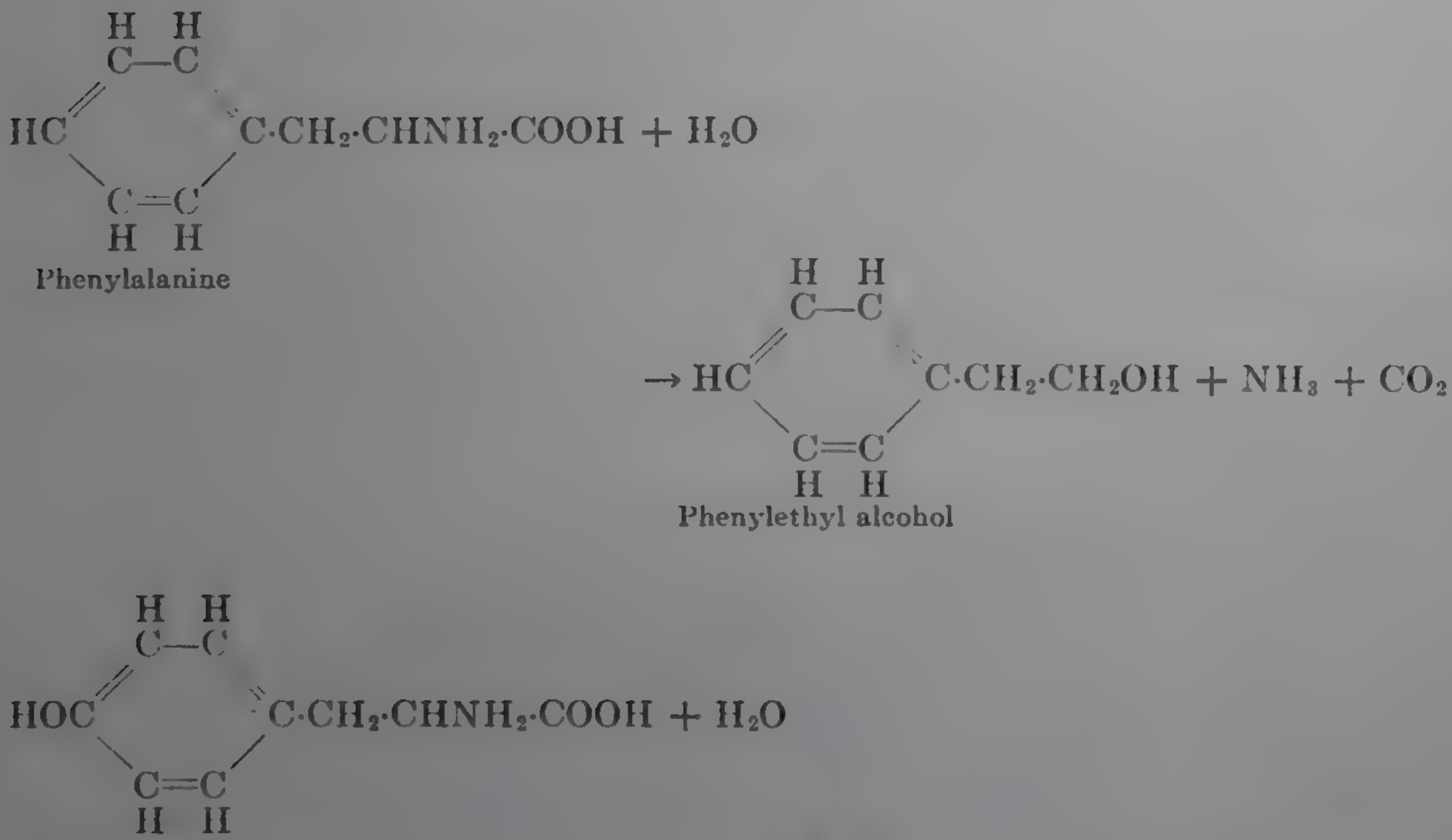
The reactions for the conversion of certain amino acids to alcohols follow:


Leucine — Isoamyl alcohol

[1] BURK, D., and C. K. HORNER, *Wallerstein Labs. Communs.*, No. 6: 5–23 (1939).

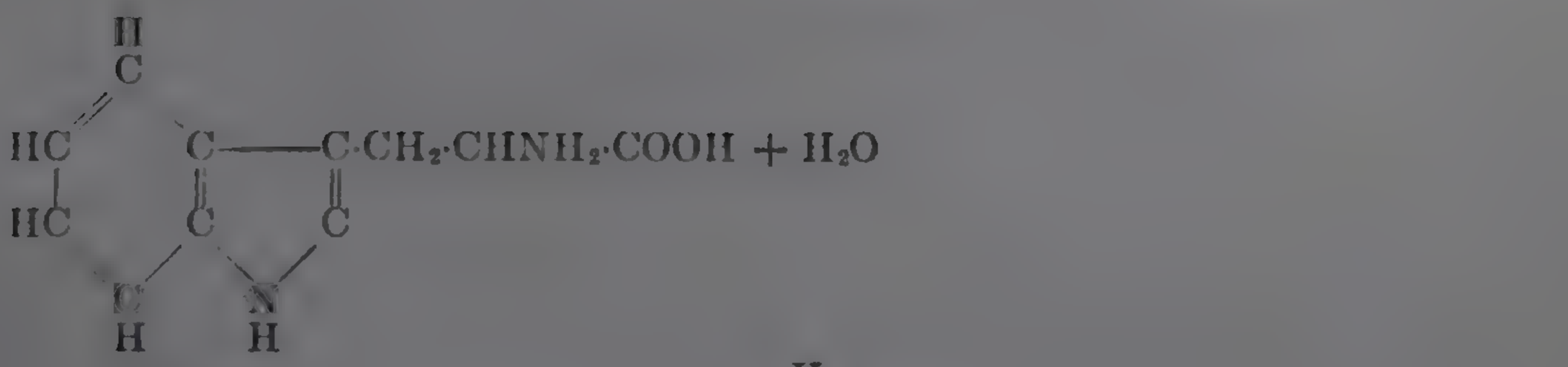


Isoleucine — Amyl alcohol



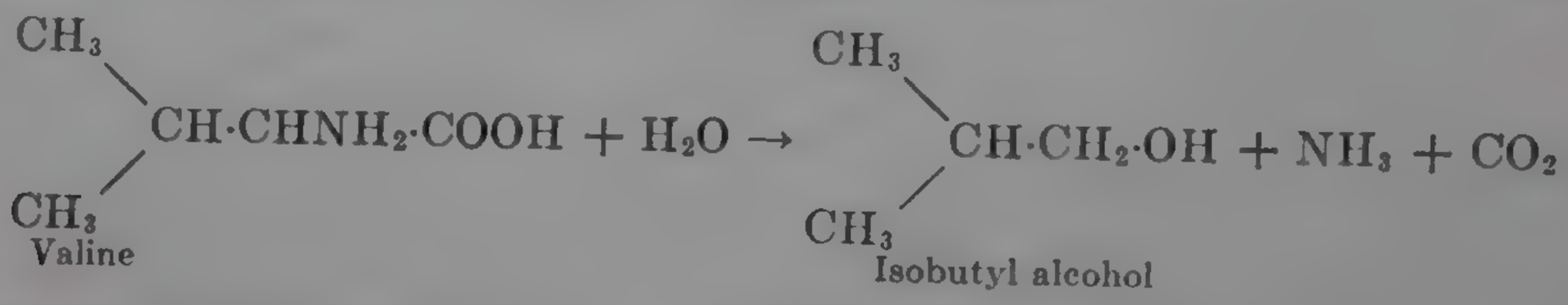
Phenylalanine — Phenylethyl alcohol

Tyrosine — Tyrosol



Tryptophane — Tryptophol

Thorne[1] has shown that isobutyl alcohol may be formed from valine by yeast in accordance with the following reaction:



The ammonia liberated by any of the foregoing reactions is available for utilization by yeasts. The reactions explain the origin of some of the constituents of fusel oil, such as amyl alcohol, isoamyl alcohol, and isobutyl alcohol.

EFFECT OF VITAMINS ON NITROGEN ASSIMILATION.—The ability or the inability of a given species of yeast to utilize a particular nitrogen source has been used as one means of classifying yeasts. Stelling-Dekker[2] employed potassium nitrate in classifying the sporogenous yeasts. Later, Lodder[3] used additionally ammonium sulphate, asparagine, peptone, and urea as aids in the classification of asporogenous yeasts. In order to determine the ability of the yeasts to utilize a particular nitrogen compound, she used a modification of the auxanographic method. A large inoculum of yeast was employed to supply the growth-promoting substances required.

Lodder found that the majority of yeasts examined by her were able to utilize the nitrogen-containing compounds tested, but that all the species of *Kloeckera* and some of those of *Torulopsis* were unable to utilize ammonium sulphate, asparagine, and urea.

Studies of the assimilation of nitrogen compounds by species of *Candida* have led to contradictory results, particularly in respect to the utilization of ammonium sulphate, asparagine, peptone, and urea.[4,5,6]

Wickerham[7] carried out tests with various species of yeasts reputedly unable to utilize ammonium sulphate, urea, and asparagine. He demon-

[1] THORNE, R., *Jour. Inst. Brewing*, **43**: 288 (1937).

[2] STELLING-DEKKER, N. M., Die Hefesammlung des Centraalbureau voor Schimmel-cultures. I Teil. Die sporogenen Hefen, *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II, Deel **28**: 1–547 (1931).

[3] LODDER, J., Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II, **32**: 1–256 (1934).

[4] LANGERON, M., and P. GUERRA, *Ann. parasitol. humaine et comparée*, **16**: 162–179 (1938).

[5] DIDDENS, H. A., and J. LODDER, Die Hefesammlung des Centraalbureau voor Schimmelcultures. II Teil. Die anaskosporogenen Hefen, Zweite Hälfte, N. V. Noord-Hollandsche Uitgeversmaatschappij, Amsterdam, 1942.

[6] MACKINNON, J. E., and R. C. ARTAGAVEYTIA-ALLENDE, *Jour. Bact.*, **49**: 317–333 (1945).

[7] WICKERHAM, L. J., *Jour. Bact.*, **52**, (No. 3): 293 (1946).

strated that the strains of the species tested (obtained from the Northern Regional Research Laboratory stock collection) were able to utilize all three of these nitrogen compounds when his special medium containing vitamins and certain trace elements was used. He also showed that urea in 0.092 per cent concentration inhibited the growth of all but 1 of the 10 species of *Candida* tested. However, urea in a concentration of 0.046 per cent was assimilated by all 10 species.

The results obtained by Wickerham are of great significance, particularly in respect to the classification of yeasts, for they show that in the presence of vitamins, yeasts may assimilate nitrogen sources which they otherwise might not.

Wickerham devised and used the following medium (shown in Table 5), which includes trace elements and eight pure vitamins. This medium was used in both solid and liquid forms.

TABLE 5.—COMPOSITION OF WICKERHAM'S SPECIAL MEDIUM[1]

Ingredients	Concentration	Ingredients	Concentration
Trace elements		Vitamins	
Boron, as H_3BO_3	0.01 p.p.m.	Biotin	2 μg.*
Copper, as $CuSO_4 \cdot 5H_2O$	0.01 p.p.m.	Calcium pantothenate	400
Iodine, as KI	0.10 p.p.m.	Inositol	2,000
Iron, as $FeCl_3 \cdot 6H_2O$	0.05 p.p.m.	Niacin	400
Zinc, as $ZnSO_4 \cdot 7H_2O$	0.07 p.p.m.	*p*-Aminobenzoic acid	200
		Pyridoxine hydrochloride	400
		Thiamin hydrochloride	400
		Riboflavin	200
Salts		Carbon source	
KH_2PO_4	0.875 g.*	Glucose	10.0g*
K_2HPO_4	0.125 g.		
$MgSO_4 \cdot 7H_2O$	0.50 g.	Nitrogen sources[2]	
NaCl	0.10 g.	$(NH_4)_2SO_4$	1.00g*
$CaCl_2 \cdot 2H_2O$	0.10 g.	KNO_3	0.78
		Urea	0.46
		Asparagine	1.00
		Peptone	1.32

[1] WICKERHAM, L. J., *Jour. Bact.*, **52** (No. 3): 293 (1946).
[2] Used separately.
* Units given in terms of a liter of solution.

Mineral Requirements.—The exact mineral requirements of yeasts in many cases are not fully known. The literature contains some conflicting statements on the subject, as has been shown by Joslyn[1] in his extensive

[1] JOSLYN, M. A., *Wallerstein Labs. Communs*, **4** (No. 11): 49 (1941).

review of the mineral metabolism of yeasts. Further careful research in this field should lead to a better understanding of the subject and practical improvements in the selection of media for growth, sporulation, and fermentation.

Chemical and spectroscopic analyses of yeasts and the media in which they are grown furnish considerable valuable information regarding the indispensability of the elements. However, the mere fact that an element is found in the ash of yeast in small amounts does not establish its essentiality.

The following table presents data concerning the composition of yeast ashes as determined by three different laboratories.

TABLE 6.—COMPOSITION OF YEAST ASHES[1,2]

Ash constituent	Top yeast[3]	Bakers' yeast[4]	Bakers' yeast[5]
P_2O_5	52.3	52.3	54.5
K_2O	35.4	35.4	36.5
Na_2O	0.06	0.60	0.7
MgO	4.8	4.8	5.2
CaO	1.56	1.56	1.4
SiO_2	1.1	1.1	1.2
SO_3	0.41	.....	0.5
Cl	.....	.....	Trace
FeO	0.43	0.34	Trace

[1] JOSLYN, M. A., *Wallerstein Labs. Communs.*, **4** (No. 11): 49 (1941).
[2] In percentage.
[3] FULMER, *et al.* (1928).
[4] HELLER (1925).
[5] FREY (1930).

A study of this table indicates that phosphorus and potassium are present in large amounts in yeast ash, comprising about 90 per cent of the total elements. The elements magnesium, calcium, silicon, sodium, iron, and sulphur are present in considerably smaller quantities.

Phosphorus is a particularly important element in the life processes of yeasts. It plays a very significant role in the production of ethyl alcohol from sugars as, for example, in the formation of hexose and triose phosphates. It is a component of cozymase and of cocarboxylase (an activator of the enzyme carboxylase). It is found in nucleic acid, in lecithin, and in other components of the yeast cell.

Natural media and worts normally contain sufficient of the inorganic elements for good growth. However, in studying the effect of a given element or growth-promoting substance on growth or fermentation, it is in most cases essential to know the exact composition of the medium employed. Ordinary chemical analysis often fails to reveal sufficient

TABLE 7.—ELEMENTS PRESENT IN YEAST ASHES AND IN THE COMPONENTS OF THE CULTURE MEDIA[1,2]

Element	Ashes from yeast grown in					Ashes from components of media						
	Williams' medium		Malt extract	Yeast extract	Autolyzed yeast	Asparagine	Asparagine mother liquor	$(NH_4)_2SO_4$	KH_2PO_4	$CaCl_2$	$MgSO_4$	Malt extract
	Standard	Enriched										
Al	—	—	—	—	++	+	+	—	—	—	—	—
Ba	+	+	+	+	?	+	+	+	—	+	—	+
Be	—	—	—	—	?	—	—	—	—	—	—	—
Bi	—	+	?	+	+	—	—	—	?	—	—	—
B	—	—	++	++	++	++	++	—	?	—	—	++
Ca	+	+	+	+	+	+	+	?	?	[3]	—	+
Cr	+	+	+	+	+	+	+	+	+	—	—	+
Cu	++	++	++	++	++	++	++	—	—	—	—	++
Au	+	+	+	+	?	+	+	—	+	—	—	+
Fe	+	+	+	+	+	+	+	+	+	+	—	+
La	+	+	+	—	?	—	+	—	—	—	—	+
Pb	+	+	++	—	+	+	+	?	—	—	—	?
Li	—	—	—	—	—	+	?	—	—	+	—	+
Mg	+	+	+	+	+	+	+	—	—	—	[3]	+
Mn	++	+	++	++	++	++	++	—	—	—	?	++
P	+	+	+	+	+	+	+	—	[3]	—	—	+
K	+	+	+	+	+	+	+	—	[3]	—	—	+
Rb	?	?	?	—	—	?	?	—	—	—	—	?
Pt	+	+	+	—	+	+	+	—	—	—	—	—
Si	+	—	++	++	++	++	++	—	—	—	—	+
Ag	+	+	+	+	+	+	+	—	?	?	—	—
Sr	—	—	+	++	+	+	++	—	—	+	—	+
Tl	+	++	+	?	+	+	+	—	—	—	—	—
Sn	+	++	++	++	++	++	++	?	+	—	—	+
W	—	—	—	—	—	?	?	—	—	?	—	?
V	—	?	—	?	?	—	—	—	—	—	—	—
Zn	?	—	+	—	—	+	++	—	—	—	—	?

[1] RICHARDS, O. W., and M. C. TROUTMAN, *Jour. Bact.*, **39** (No. 6): 739 (1940).
[2] + = present, ++ = strongly present, — = absent, ? = possible trace.
[3] Known part of medium.

information concerning the trace elements present. Such information may be supplied by the use of a spectroscope. Richards and Troutman[1] have analyzed spectroscopically the mineral content of the ashes from yeasts grown in certain culture media, and of the ashes from the components of the media. Table 7 presents data obtained by them.

Certain trace elements, for example, copper, iron, thallium, and man-

[1] RICHARDS, O. W., and M. C. TROUTMAN, *Jour. Bact.*, **39**: 739 (1940).

ganese, are of considerable importance. Elvehjem[1] demonstrated that bakers' yeast will grow poorly in a medium low in iron and copper. These elements appear to be essential for the elaboration of cytochrome. Iron catalyzes respiration through the action of catalase, peroxidase, and cytochrome oxidases. According to Richards,[2] growth of yeast is stimulated when thallium is added to Williams' medium (20 g. of sucrose, 3 g. of $(NH_4)_2SO_4$, 2 g. of KH_2PO_4, 1.5 g. of asparagine, 0.25 g. of $CaCl_2$, and 0.25 g. of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water). Edbacher is of the opinion that manganese activates yeast arginase.

Further information concerning the mineral metabolism of yeasts and methods of studying the nutrition of yeasts will be found in the following paragraphs.

TABLE 8.—SOME SEMISYNTHETIC MEDIA

Substance	Medium of Devereux and Tanner,[1] grams	Medium F of Fulmer and Nelson,[2] grams	Medium of Mayer,[3] grams
Sucrose	10.0	10.0	15.0*
Dextrin	.....	0.60	
NH_4Cl	0.12	0.188	
K_2HPO_4	0.05	0.100	
KH_2PO_4	.....	.....	0.1
$CaCl_2$	0.01	0.100	
$Ca_3(PO_4)_2$	.....	.....	0.1
$MgSO_4$	0.02	.....	0.1
Distilled water	100.00 cc.	100.0 cc.	100.0 cc.

[1] DEVEREUX, E. D., and F. W. TANNER, *Jour. Bact.*, **14**: 317 (1927).

[2] Formula for incubation at 30°C. F. W. Tanner, E. D. Devereux, and F. M. Higgins, *Jour. Bact.*, **11**: 45 (1926).

[3] GUILLIERMOND, A., "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

*Calculated.

Media.—Food materials are supplied to yeasts in the form of media (singular, medium) which contain all the essential materials necessary for growth, reproduction, or fermentation. Media may be synthetic, semisynthetic, or nonsynthetic in nature. A synthetic medium is one in which the composition of all the ingredients is known. A semisynthetic medium is one containing some constituents of known composition and some whose analysis is only approximately known. A nonsynthetic medium is one whose exact composition is not known. The composition of chemically pure salts, sugars, acids, and certain other chemical compounds is definitely established, whereas that of such items

[1] ELVEHJEM, C. A., *Jour. Biol. Chem.*, **90**: 111 (1931).

[2] RICHARDS, O. W., *Jour. Am. Chem. Soc.*, **47**: 1671 (1925).

as beef extract and peptone are more qualitative than quantitative. Media such as apple juice and grape juice are natural sources of the required food materials for the yeasts, as is evident by the fact that the yeasts found on the surfaces of apples and grapes rapidly ferment at optimum temperatures the juices expressed from them.

Media favorable for the growth of yeasts are also suitable for the growth of molds. Owing to the rather acid nature of these media many bacteria fail to develop well in them or are inhibited entirely.

For a further discussion of the subject of yeast nutrition, the reader is referred to Guilliermond's or other standard texts.

Relative Rates of Fermentation of Glucose and Fructose.—Not all sugars are fermented at the same rate. Nor do all yeasts act with equal efficiency. In low concentrations of sugar, fructose is fermented at a slower rate (expressed as milligrams of carbon dioxide per minute) than glucose by brewers' yeast at 30°C.[1] Although the maximum rates for the fermentation of these two sugars differ but little, the rate for glucose is slightly greater than that for fructose at the same concentration. Glucose is fermented at about the same rate in concentrations between 1 and 10 per cent; fructose, between 2 and 8 per cent. At high concentrations of the sugars, the rate of fermentation of fructose is less than that of glucose. This is probably referable to the difference in the ease with which the enzymes involved can bring about the cleavage of the sugar molecule.

Yeast Enzymes.—The enzymes present in yeast include, usually, at least three different groups: those concerned with respiration; those concerned with breaking sugars down to the hexose stage, for example, maltase and invertase; and finally those concerned with fermentation.

Enzymes catalyze the complex chemical changes that take place in nutrient sugar solutions. Whether or not a carbohydrate is fermented or assimilated depends on the nature of the enzymes elaborated by the yeast, provided, of course, that conditions are otherwise favorable for fermentation or growth. Polysaccharides, in general, are not fermented. However, Wickerham and associates[2] found that *Endomycopsis fibuliger* possessed an extracellular amylase system with a high alpha- to beta-amylase ratio. Lactose is fermented by *Saccharomyces fragilis* (*S. kefir*), the yeast of the fermented milk product, kefir, and by a few other yeasts but not by *S. cerevisiae* and *S. ellipsoideus*, which represent the best-known and probably most widely distributed types of yeast.

[1] HOPKINS, R. H., and R. H. ROBERTS, Kinetics of Alcoholic Fermentation of Sugars by Brewer's Yeast, *Biochem. Jour.*, **29**: 931 (1935).

[2] WICKERHAM, L. J., L. B. LOCKWOOD, O. G. PETTIJOHN, and G. E. WARD, *Jour. Bact.*, **48** (No. 4): 413 (1944).

The enzymes of yeasts are of two kinds: endoenzymes (intracellular) and exoenzymes (extracellular). These enzymes react according to the general laws governing enzyme reactions but show also a degree of specificity in each case.

The enzymes of yeasts may be classified as hydrolases and desmolases.

Hydrolases are enzymes that convert carbohydrates, proteins, and esters to simpler substances by the addition of water followed usually by cleavage. For example, sucrose is converted, after the absorption of water, to 1 molecule each of glucose and fructose as a result of the action of sucrase, an enzyme; while raffinose, a trisaccharide, is converted to 1 molecule each of fructose, glucose, and galactose through the action of melibiase and sucrase, enzymes possessed by "bottom" yeast.

Glycogen is hydrolyzed to glucose, but no cleavage follows the addition of the molecule of water to the glycogen molecule. Although glycogenase may thus convert glycogen to glucose, it may, under certain conditions, cause the reaction to go in the opposite direction. Thus, some enzymes have the ability to catalyze reversible reactions and so to synthesize compounds as well as to break them down to simpler components.

Desmolases are enzymes or enzyme complexes involved in respiration and metabolism. Zymase is a desmolase of intracellular origin and rarely or never passes through the cell wall into the surrounding medium.

Zymase is not a single enzyme, but a complex of enzymes and coenzymes. The term "holozyme" has been coined to include the zymase complex plus all its activators. Hexokinase, oxidoreductase, enolase, carboxylase, and phosphatase are said to be some of the enzymes of this complex.

Table 9 summarizes data concerning some of the enzymes found in yeasts, the substrates acted upon, and the end products formed as a result of this action.

Coenzymes and Activators.—A coenzyme, phosphate, magnesium, or some other substance may be essential in order that an enzyme may function properly. Cozymase (coenzyme I) is required for the action of oxidoreductase; cocarboxylase, for carboxylase, the enzyme which splits carbon dioxide from such alpha-ketonic acids as pyruvic acid. Magnesium is essential for the activation of phosphatase.[1] Glutathione is required by methylglyoxalase.

Cell-free yeast juice from crushed or autolyzed cells will ferment sugar but more slowly than yeast cells, a fact demonstrated by Buchner. If yeast juice is dialyzed by passing it through a semipermeable membrane, neither the dialyzate, the portion passing through the membrane,

[1] TAUBER, H., "Enzyme Chemistry," John Wiley & Sons, Inc., New York, 1937.

TABLE 9.—SOME ENZYMES OF YEASTS, SUBSTRATES ACTED UPON, AND PRODUCTS FORMED

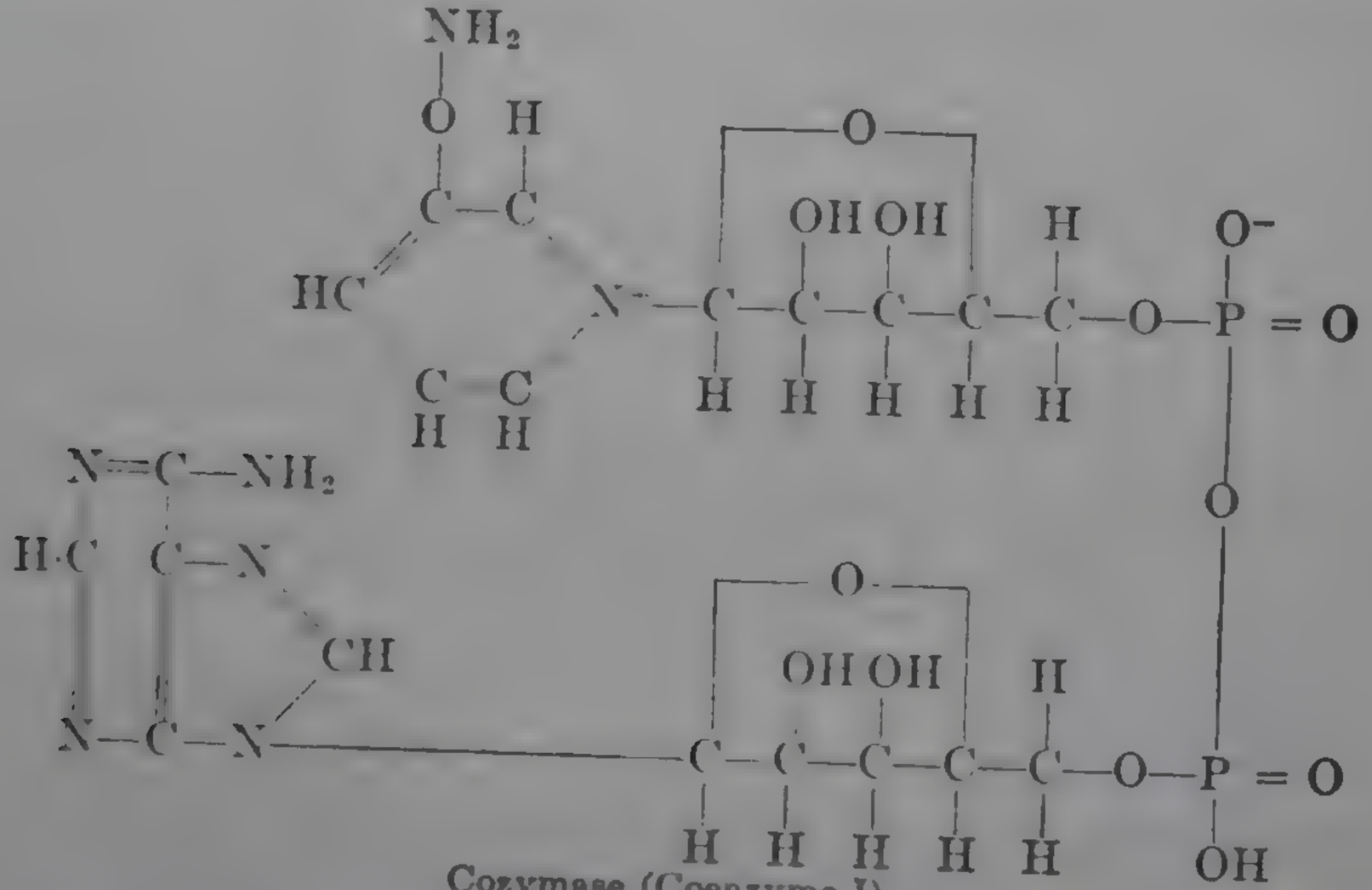
Enzyme	Substrate	Products formed
I. Hydrolases:		
A. Carbohydrases:		
1. Sucrase (Saccharase, invertase, invertin)	$C_{12}H_{22}O_{11}$ Sucrose	$C_6H_{12}O_6$ Glucose + $C_6H_{12}O_6$ Fructose
2. Maltase	$C_{12}H_{22}O_{11}$ Maltose	$2C_6H_{12}O_6$ Glucose
3. Lactase	$C_{12}H_{22}O_{11}$ Lactose	$C_6H_{12}O_6$ Galactose + $C_6H_{12}O_6$ Glucose
4. Melibiase	$C_{12}H_{22}O_{11}$ Melibiose	$C_6H_{12}O_6$ Galactose + $C_6H_{12}O_6$ Glucose
5. Trehalase	$C_{12}H_{22}O_{11}$ Trehalose	$2C_6H_{12}O_6$ Glucose
6. Glycogenase	$(C_6H_{10}O_5)_x$ Glycogen or $C_6H_{12}O_6$ Glucose	$C_6H_{12}O_6$ Glucose $(C_6H_{10}O_5)_x + xH_2O$ Glycogen
B. Proteolytic enzymes:		
1. Proteases	Yeast proteins	Proteoses, peptones, and polypeptides
2. Peptidases	Peptides	Amino acids
C. Esterases:		
1. Phosphatases:		
a. Polynucleotidase	Nucleic acid	Mononucleotides
b. Phosphatese	Hexose + H_3PO_4	Hexosephosphate
D. Amidases:		
1. Asparaginase	$H_2N{\cdot}CO{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$ Asparagine	$HOOC{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH + NH_3$ Aspartic acid
II. Desmolases:		
A. Zymase group:		
1. Oxydoreductase (Mutase, dehydrase)	RCHO Aldehyde	RCH_2OH Alcohol + RCOOH Acid
2. Glycerolphosphoric dehydrogenase	Glycerolphosphoric acid	Glyceraldehyde phosphoric acid
3. Carboxylase	$CH_3{\cdot}CO{\cdot}COOH$ Pyruvic acid	$CH_3{\cdot}CHO$ Acetaldehyde + CO_2 Carbon dioxide
4. Methylglyoxalase	$CH_3CO{\cdot}CHO$ Methylglyoxal	$CH_3CHOHCOOH$ Lactic acid
5. Hexokinase	Hexoses	Active hexoses

nor the residue will produce fermentation of sugar. Should the two fractions be mixed, however, fermentation ensues.

The residue, the portion not passing through the semipermeable membrane, is thermolabile, being destroyed by boiling. The non-dialyzable enzymes are found in this fraction.

Cozymase.—The dialyzate is thermostable and contains, in addition to other substances, cozymase or coenzyme I.

Euler and Schlenk[1] have proposed the following structural formula for cozymase or coenzyme I.



Cozymase (Coenzyme I)

Euler and others believe that cozymase is a hydrogen-carrying coenzyme, which picks up 2 atoms of hydrogen (to form dihydrocozymase) and later donates them. It acts as an intermediate between two apodehydrases in the transportation of hydrogen from the donator system (glyceraldehyde phosphoric acid-phosphoglyceric acid) to the acceptor system (acetaldehyde-ethyl alcohol) in the ethanol fermentation.[2] (For further details concerning the function of cozymase, refer to page 157.)

Cozymase may be prepared by dialyzing fresh yeast juice, which contains about 0.5 g. of cozymase per kilogram of juice,[3] or by washing zymin with water.

The coenzyme may be isolated from most animal tissues; from many plants and fungi; from certain bacteria, namely, those giving rise to propionic and lactic acids; and from red blood corpuscles.

The Adenylic Acid System.—The dialyzate of yeast juice also contains a phosphate carrier system, which functions as a coenzyme. This

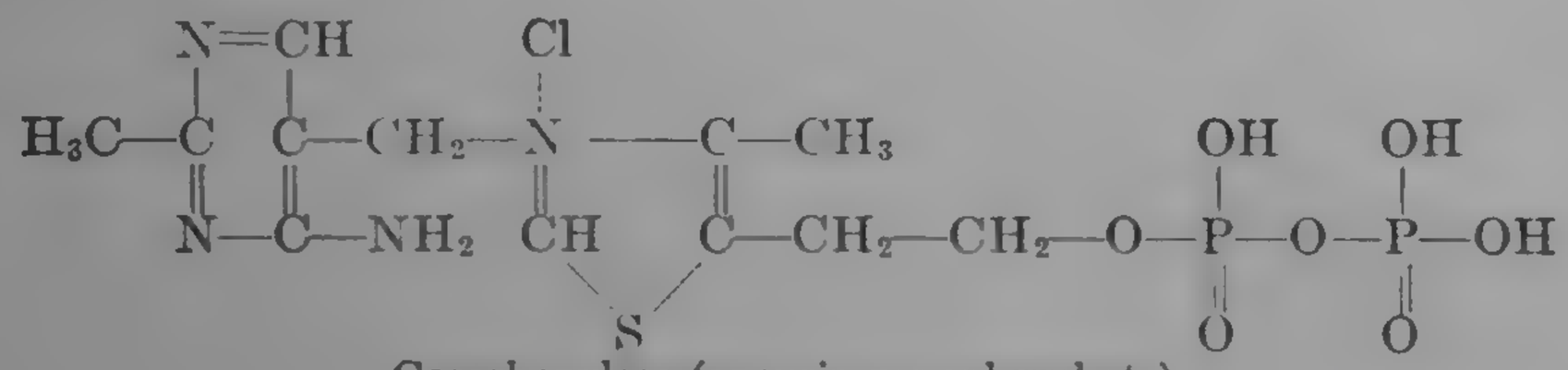
[1] Lohmann, K., The Chemistry and Metabolism of the Compounds of Phosphorus, *Ann. Rev. Biochem.*, **7**: 125 (1938).

[2] Euler, H. von, *Angew. Chem.*, **50**: 831 (1937).

[3] Meyerhof, O., und P. Ohlmeyer, *Biochem. Zeit.*, **290**: 334 (1937).

system is made up of adenosinemonophosphate (adenylic acid), adenosinediphosphate and adenosinetriphosphate. For further details, consult the chapter on Facts and Theories Concerning the Mechanism of the Ethanol Fermentation.

Cocarboxylase.—Lohmann and Schuster[1] have assigned the following structural formula to the coenzyme cocarboxylase, which is diphosphorylated vitamin B:



Cocarboxylase (aneurin pyrophosphate)

Cocarboxylase is thermostable and is found in yeast juice. When beer yeast is washed with an alkali, it can no longer decarboxylate pyruvic acid, but the addition of boiled, fresh beer-yeast juice causes the system to become active again, according to the researches of Anhagen,[2] who called the thermostable principle "cocarboxylase."

Phytochemical Reduction.—Yeast possesses strong reducing properties.

A relatively large number of substances may be added to an actively fermenting medium, containing yeast and sugar, and become reduced. Aldehydes are reduced to their corresponding alcohols; for example, acetaldehyde, glyceraldehyde, furfuraldehyde (furfural), and benzaldehyde are reduced to ethyl alcohol, glycerol, furfuralcohol, and benzyl alcohol, respectively. Ketones and diketones may be reduced to their corresponding secondary alcohols, likewise, but the rate of reduction is slower than it is for aldehydes.[3]

The reducing action of yeast is not confined, however, to aldehydes and ketones alone, for methylene blue may be reduced; aniline may be formed from nitrobenzene, and hydrogen sulphide from sulphur. Other types of substances may become reduced also.

Reduction may be readily demonstrated by adding the substance to be reduced to a fermenting medium containing 10 per cent sucrose and 10 per cent living yeast.

[1] Lohmann, K., und P. Schuster, *Naturwissenschaften*, **25:** 26 (1937); *Biochem. Zeit.*, **294:** 188 (1937).

[2] Anhagen, E., *Zeit. physiol. Chem.*, **204:** 149 (1932); *Biochem. Zeit.*, **258:** 330 (1933).

[3] Harden, A., "Alcoholic Fermentation," 4th ed., Longmans, Green & Company, New York, 1932.

Classification of Yeasts.—The problem of classification of the yeasts is one of great difficulty, and modern work has not tended to eliminate all the confusion that has previously existed. The most recent classification, here given, supersedes that published in Guilliermond's treatise on this group of fungi but agrees with it in putting practically all the yeasts now industrially important in the genus *Saccharomyces*.

The following classification is that of Stelling-Dekker, modified in some respects by Guilliermond.[1]

FAMILY OF ENDOMYCETACEAE

I. Subfamily of the *Eremascoideae*.

Genus *Eremascus* Eidam.

Thallus formed by a mycelium without any form of asexual multiplication. Asci formed by isogamic conjugation, 8 round ascospores.

II. Subfamily of the *Endomycetoideae*.

Thallus formed by a typical mycelium multiplying by means of oïdia or thallus reduced to the state of oïdia.

1. Genus *Endomyces* Reess.

Typical mycelium multiplying by oïdia.

Asci formed by heterogamic conjugation or by parthenogenesis; 4 round or hat-shaped ascospores.

2. Genus *Schizosaccharomyces* Lindner.

Thallus reduced to the state of oïdia. Asci formed by isogamic conjugation; 4 to 8 round ascospores.

III. Subfamily of the *Saccharomycoideae*.

Thallus formed by a typical mycelium multiplying by means of conidial buds and occasionally at the same time by oïdia, or thallus reduced to the state of yeast cells.

A. Tribe of the *Endomycopseae*.

Thallus formed by a typical mycelium multiplying by means of conidia yeasts conidial buds) and occasionally at the same time by oïdia. Asci formed by heterogamic conjugation or by parthenogenesis, occasionally after rudimentary attempts of union (vestiges of sexuality). Ascospores in the form of a hat, sickle, or girdled in the middle by a projecting filament, smooth or sometimes verrucose.

1. Genus *Endomycopsis* Dekker.

B. Tribe of the *Saccharomyceteae*.

Thallus reduced to yeast cells that are able occasionally to produce rudimentary mycelia.

1. Genus *Zygosaccharomyces* Barker.

Cells round, oval, or elongated. Asci formed by iso- or heterogamic conjugation: 1 to 4 round or reniform ascospores. Fungi developing in liquid media first in the form of a deposit and producing a pellicle or ring only slowly or not producing any. Producers of alcoholic fermentations.

[1] GUILLIERMOND, A., La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents, Masson et Cie, Paris, 1937.

2. Genus *Torulaspora* Lindner.

Round cells. Ases formed by parthenogenesis in cells having tried to conjugate; 1 to 2 round and smooth ascospores. Fungi vegetating in the form of a deposit and not producing pellicle or ring or producing it only slowly.

3. Genus *Saccharomyces* Meyen.

Round oval, or elongated cells, at times with rudimentary mycelia. Ases formed without conjugation; 1 to 4 round and smooth ascospores. Conjugation may be produced between the ascospores or more generally between the first cells produced by their budding; zygospores being the point of departure from diploid cells which, after having produced numerous generations, are transformed into ases. Fungi vegetating first in liquid media in the form of a deposit. Film or ring formed slowly or not at all. Producers of fermentations.

4. Genus *Pichia* Hansen.

Cells oval or elongated. Ases formed by iso- or heterogamic conjugation or by parthenogenesis; 1 to 4 hemispherical, reniform, or triangular smooth ascospores. Fungi forming, from the beginning, a film on the surface of liquid media. Oxidations, sometimes fermentations.

a. Subgenus *Zygopichia* Klöcker.

Ascs derived from iso- or heterogamic conjugation.

b. Subgenus *Pichia* Hansen.

Ascs formed by parthenogenesis.

5. Genus *Hansenula* (*Willia* Klöcker) Sydow.

Yeasts oval or elongated, rarely round, at times rudiments of mycelium. Ases formed without conjugation; 1 to 4 ascospores having the appearance of a hat or girdled with a protruding ring. In certain forms (*Hansenula Saturnus*), conjugation among the ascospores, or more generally among the first cells derived from their budding, and producing zygospores, point of departure of numerous generations of diploid cells being transformed finally into ases. Fungi developing from the beginning on liquid media, without film formation. Oxidations and sometimes fermentations.

6. Genus *Debaryomyces* Klöcker.

Round or oval cells, sometimes rudimentary mycelia. Ases formed by iso- or heterogamic conjugation; 1 to 2, sometimes 4, round and globular ascospores. On liquid medium, first a deposit, then film or ring. Sometimes producers of fermentations.

7. Genus *Schwanniomyces* Klöcker.

Round or oval cells, occasionally rudimentary mycelia. Ases formed by parthenogenesis after attempts of union (vestiges of sexuality); 1 to 2 ascospores provided with a medium of a projecting filament and with a verrucose wall.

C. Tribe of *Nadsonieae*.

Cells elongated and ordinarily apiculate and producing new cells at either pole by a process intermediate between budding and division.

1. Genus *Nadsonia* Sydow (*Guilliermondia* Nadson and Konokotine).

Ases formed in a bud derived from a zygote resulting from a heterogamic conjugation; 1 to 2, sometimes up to 4, round and verrucose ascospores. Vegetation on liquid medium, in form of a deposit and with production of alcohol.

2. Genus *Saccharomycodes* Hansen.

Ases with 4 round and smooth ascospores. Conjugation produced regularly between the ascospores. Vegetation in liquid media first in form of a deposit. Produce fermentations.

1. Genus *Hanseniaspora* Zikes (*Hansenia* Lindner).

Cells only slightly ogival. Asci formed without conjugation, 1 to 4 hemispherical or round ascospores.

IV. Subfamily of *Nematosporoideae*.

Cells of variable forms and often in pseudomycelium. Asci with 1 to 8 ascospores in form of long needles.

1. Genus *Nematosporomyces* Matrucchot.

Oval yeasts. Asci with 1 ascospore in form of long needle.

2. Genus *Nematospora* Peglion.

Yeasts of variable form and mycelium; 1 to 8 ascospores in form of needles provided at one of their poles with a sort of long flagellum.

3. Genus *Coccidiascus* Chatton.

Oval yeasts. Asci appearing to be derived from an isogamic conjugation; 8 ascospores in the form of long needles.

The ascosporeforming (sporogenous) yeasts have been classified by Stelling-Dekker (1931); and the nonascosporeforming (asporogenous, or anascosporogenous) yeasts, by Lodder (1932, 1934) and by Diddens and Lodder (1942). Individual genera have been considered by Custer (1930), who studied the genus *Debaryomyces*; by Bedford (1942), who made a taxonomic study of the genus *Monilia*; by Nickerson (1944), who studied the genus *Zygosaccharomyces*; and by others.

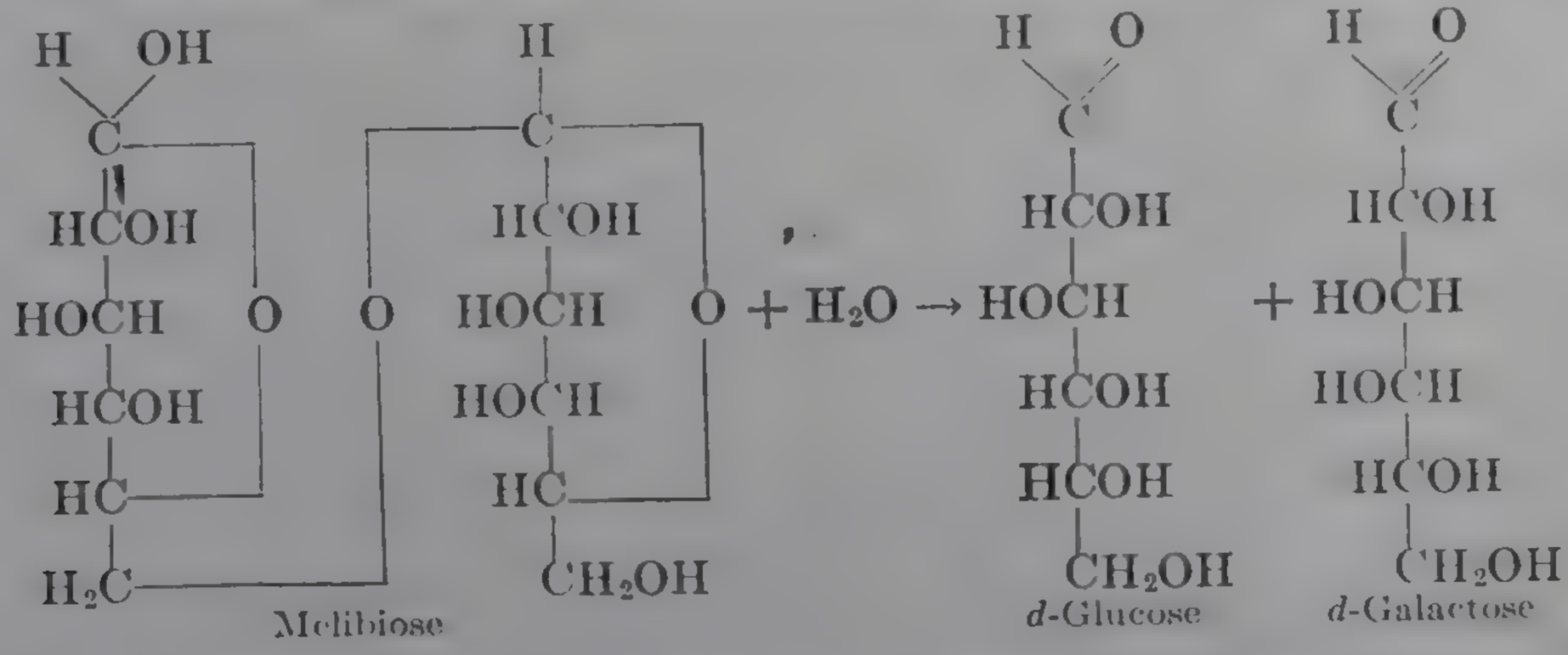
Information concerning classification may be found in a number of works other than those mentioned above. The comprehensive reviews of Rivalier (1941) and of Skinner (1947), who considered the yeast-like fungi (Endomycetales and *Fungi Imperfecti*), are particularly noteworthy. Valuable data are to be found in "Zymologia Medica," by Mackinnon (1946); and in "Hospital Mold, Yeasts and Actinomycetes" by Skinner, Emmons, and Tsuchiya (1947).

According to Schultz and Atkin (1947), the bios requirements of different strains of yeast may be used as a basis for classifying them. In order to determine the bios requirements, the yeast is grown in a special synthetic medium to which is added casein-hydrolyzate and growth factors. The latter are numbered as follows: (1) inositol, (2) calcium pantothenate, (3) biotin, (4) thiamin, (5) pyridoxine, and (7) nicotinic acid. The bios requirement is placed as a number after the species name of the yeast. For example, *Saccharomyces cerevisiae* 23 indicates that this particular strain requires calcium pantothenate and biotin for full growth.

The Fermentation of Raffinose and Melibiose.—The ability of certain yeasts to attack raffinose and melibiose, or their inability to attack them, has been used as a differential test and as an aid in classification; for

example, Stelling-Dekker used this test in classifying the genus *Saccharomyces*.

Raffinose is a trisaccharide that structurally is a combination of melibiose and sucrose with the glucose radical common to the two. Upon partial hydrolysis by sucrase it is converted to melibiose and fructose, the enzyme sucrase acting only on the sucrose portion of the trisaccharide. Melibiose (glucopyranose-6-α-galactopyranoside) is a disaccharide, which may be converted by the enzyme melibiase to glucose and galactose.



Yeasts, which secrete both sucrase and melibiase, hydrolyze raffinose to 1 molecule each of fructose, glucose, and galactose. Such yeasts completely ferment raffinose. Other yeasts, which possess sucrase but not melibiase, hydrolyze raffinose to melibiose and fructose. These yeasts ferment only fructose or one-third of the raffinose molecule.

A number of procedures have been devised to determine the extent to which raffinose is fermented. These involve the use of a quantitative apparatus, such as the van Iterson-Kluyver apparatus;[1] the absorption of the carbon dioxide produced during fermentation by means of barium hydroxide and the titration of the excess alkali with a standard acid solution;[2] the titration of the unfermented sugar after 14 days;[3] and a method devised by Wickerham,[4] which is described below.

In Wickerham's procedure, a 4 per cent nutrient solution of raffinose is dispensed in special tubes and inoculated with the yeast being tested. After the fermentation has subsided and the volume of gas has commenced to decrease, each tube is inoculated with a melibiose-fermenting yeast. A record is made of the amount of gas produced by each yeast.

The basal medium contained 0.45 per cent Difco yeast extract, 0.75 per cent peptone, and sufficient brom thymol blue to produce a green color

[1] Henrici, A. T., *Bact. Revs.*, **5**: 97 (1941).

[2] "Manual of Methods for Pure Culture Study of Bacteria," Leaflet VI, Society of American Bacteriologists; Geneva, N. Y., 1942.

[3] Zimmermann, J. G., *Zentr. Bakt. Parasitenk.*, **II, 98**: 36 (1938).

[4] Wickerham, L. J., *Jour. Bact.*, **46** (No. 6): 501 (1943).

ENDOMYCETACEAE

SACCHAROMYCETACEAE

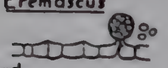
(family)

Mycelium promycelium oidia or conidia
Budding or cell division.
Iso or hetero copulation or parthenogenesis, naked asci not more than 8 spores-cylindrical, spherical, subspherical angular sickle or spindle shape. smooth, warty or bordered.
Ferm. or oxid or both

EREMASCOIDEAE

Mycelium
Cell division
Iso copulation
Oxidative

Eremascus



Oxid
Mycelium.
Cell div.-no budding.
Iso copulation.
No arthrospores.

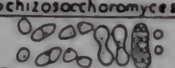
ENDOMYCOIDEAE

Mycelium and oidia or oidia only
Cell division
Iso or hetero copulation or conj between haploid cells
Oxid or ferm

Endomyces

Oxid
Mycelium & oidia.
Cell div no budding.
Round to hat shaped spores-hetero copulation or conjug between haploid cells; 4 spores per ascus.
Arthrospores.

Schizosaccharomyces



Ferm.
Oidia-no mycelium
Fission-no budding
Isogamic copulation.
4-8 round spores.

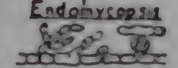
SACCHAROMYCOIDEAE

Mycelium with blastospores - or budding cells often with pseudomycelium
Fission multipolar or bipolar budding
Iso or hetero copulation or parthenogenetic spore prod
Range from purely oxid to purely ferm.

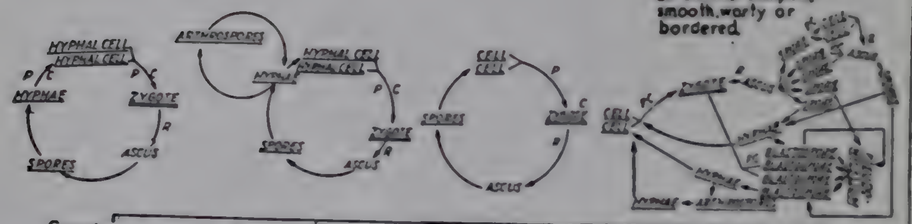
ENDOMYCOPSEAE

Mycelium, conidia, oidia at times
Cell div many sided budding.
Iso cop or conjug haploid cells.

Endomycopsis



Oxid
Mycelium & conidia, Sometimes oidia.
Cell division & many sided budding.
Blastospores possibly arthrospores
Isogamic copulation
1-4 spores, spherical semi-spherical oval or sickle shaped, smooth, warty or bordered.



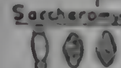
SACCHAROMYCETEAE

No mycelium, sometimes pseudomycel
Budding yeast cells
Iso or hetero copulation or conjug hapl cells

NADSONIEAE

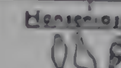
No mycelium, sometimes pseudomycel
Bipolar budding
Hetero copulation or uncertain sex process

Saccharomycodes



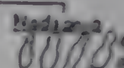
Ferm.
Lemon shaped cells
Bud fission
Hetero copulation
Spores smooth, 4 per ascus

Hanseniaspora

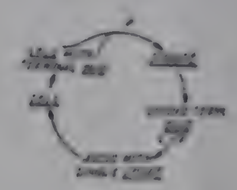


Ferm.
Bipolar budding
No mycelium
2-4 spores, round to hat shaped.

Nadsonia



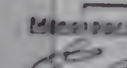
Oxid & Ferm
Egg to lemon shaped cells
Hetero copulation of mother cell & bud, second bud forms becoming ascus
One round spore



NEMATOSPOROIDEAE

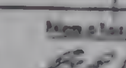
Mycelium and budding cells iso copulation, sickle or spindle-shaped spores, with or without appendages.
Oxid or ferm.

Metschnikowia



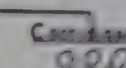
Oval cells, mycelium
Budding
Iso copulation
One spore-needle shaped.

Nematospora



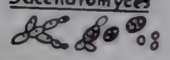
Oxid & Ferm.
Oval long, irregular or mycelium-like cells
No apparent conjug
4 or more spindle-shaped spores with appendage

Coccidiascus

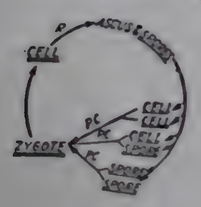


Oxid
Round to oval cells
No copulation.
8 spindle shaped spores

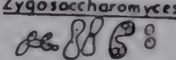
Saccharomyces



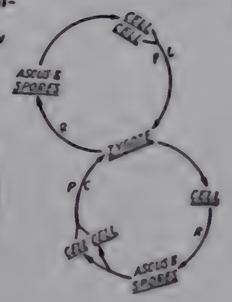
Ferm.
Round, oval to long cells.
Pseudomycelium at times.
Iso or hetero copulation or between haploid cells.
Spores round, kidney or hat shaped.



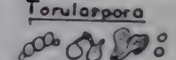
Zygosaccharomyces



Oxid & Ferm.
Spore prod after iso or hetero copulation or from diploid cell.

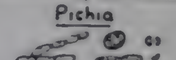


Torulaspora



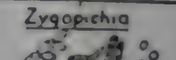
Ferm
Predominantly round cells
Abortive conjug tubes formed; no conjug.
1-2 spores-round, smooth

Pichia



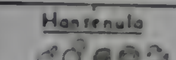
Oxid
Oval to long cells
Pseudomycelium
Weak or no ferm.
Sex process uncertain, apparently from diploid cell
1-4 spores, angular or semi spherical
Film forming
No utilization NO_3^-

Zygopichia

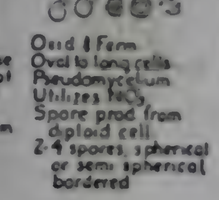


Oxid
Characteristics same as for Pichia except spore prod after iso or hetero copulation or from diploid cell

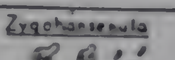
Hansenula



Oxid & Ferm
Oval to long cells
Pseudomycelium
Utilizes NO_3^-
Spore prod from diploid cell
2-4 spores, spherical or semi spherical bordered

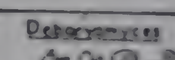


Zygohansenula

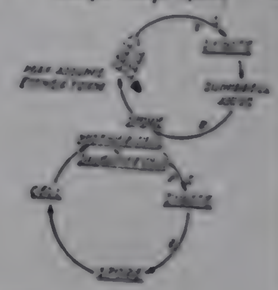


Oxid & Ferm
Characteristics as for Hansenula except spore prod from iso or hetero cop.

Debaryomyces



Oxid & Ferm
Round, sometimes oval
Pseudomycelium at times
Hetero or iso cop.
Single rough spore



Schizosaccharomyces

Ferm
Oval cells
Retort shaped asci
Abortive conjug tubes
1 or seldom 2 spores, round, warty bordered

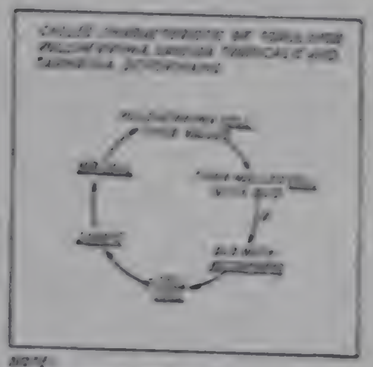


Fig. 74.—Yeast classification chart. (*Courtesy of Dr. Emil Mrak, University of California.*)

(Turning page 48.)

at a pH of 7.0. Two-milliliter portions of this medium were added to each of a series of culture tubes measuring 12 by 150 mm. and containing inverted vials measuring 6 by 50 mm. The tubes and their contents were sterilized and to each tube was added aseptically 1 ml. of a 12 per cent solution of raffinose, sterilized for 20 min. at 12 lb. steam pressure. This medium was then inoculated with a young culture of the yeast being tested, which had been grown on a yeast extract agar slant. The tubes were incubated at 30°C. Gas production was recorded from time to time, and when the amount of gas in the inverted vial commenced to decrease, the tube was inoculated with a young culture of *S. carlsbergensis* NRRL 379, an active fermenter of melibiose. The production of additional gas indicated that the test yeast had not fermented melibiose. However, failure of *S. carlsbergensis* to produce additional gas was taken as evidence that the test yeast had already fermented melibiose.

Wickerham, in surveying 200 strains of industrial yeasts, found that the average length of time required for a yeast to complete the fermentation and for the volume of the gas entrapped in the inverted vial to commence to decrease was 7 days and that the longest time required was 16 days.

Henrici[1] used culture tubes of 25 by 100 mm. size containing 20 ml. of medium and inverted Wassermann tubes (10 by 75 mm.) as gas traps in carrying out the melibiose fermentation test. He suggested that tubes inoculated with pellicle-forming yeasts be shaken a day or two after inoculation in order that some of the cells might sink to the bottom, also that all tubes be shaken before reading the final results.

Auxanographic Technique.—In order to determine whether or not a yeast is able to utilize a given sugar, Beijerinck[2] devised an auxanographic procedure. The basal medium, which contained 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulphate, 0.05 per cent ammonium sulphate, and 2.0 per cent of washed agar, was prepared, sterilized, cooled, seeded heavily with the yeast being tested while still in the melted condition, and poured into petri dishes. The heavy suspension of yeast was used in order to provide the required growth-promoting substances. Small amounts of the sugars to be tested (in the dry form) were placed upon the dry surface of the solidified agar, sufficiently far apart to prevent mixing of the dissolved materials. Glucose was used as a control, since all yeasts are able to utilize it. The petri dishes were incubated at an optimum temperature for growth, usually 25 to 28°C. The sugars diffuse into the agar and, if they are assimilated, cause growth

[1] HENRICI, *loc. cit.*

[2] BEIJERINCK, M. W., *Arch. néerland. sci.*, **23**: 367–372 (1889).

of the yeasts. No growth takes place when a sugar cannot be utilized. This procedure has been used by Lodder[1] and others.

Lodder[1] used a modification of Beijerinck's auxanographic technique in order to determine the extent to which certain nitrogen compounds were used by yeasts. The basal medium in this case contained 2 per cent glucose, 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulphate, and 2 per cent of washed agar. In carrying out the test, approximately 2 ml. of a heavy suspension of the yeast being examined were placed in each petri dish and the basal medium added. The plates were incubated at 30°C. for a number of hours in order to dry the surface of the agar. Small amounts of the nitrogen compounds (ammonium sulphate, asparagine, potassium nitrate, peptone, and urea) were then placed on the surface of the agar. The petri dishes were incubated at 25°C., and later observed to determine which sources of nitrogen supported growth.

Since the auxanographic method may produce erroneous results if a sugar or nitrogen compound diffuses over too wide an area in the petri dish, some workers have preferred to use liquid media and to test for the utilization of each given substance in separate culture tubes.

GROWTH-PROMOTING SUBSTANCES

Definition.—Growth-promoting substances, or growth-accessory factors, may be considered to be substances which, when added to a medium containing the usual sources of energy, carbon, nitrogen, and basic inorganic salts, accelerate the growth of a microorganism.

Controversial Nature.—The subject of growth-promoting substances, especially bios, has been a cause of controversy, particularly during the first part of the present century. Failure to agree on findings in different laboratories has been due in large part to the use of different species or strains of yeasts or other microorganisms. Copping,[2] Williams,[3] and others have shown that the requirement for bios, for example, depends on the type of yeast and the composition of the medium.

Bios.—Space does not permit an extensive review of the literature concerning bios. The subject was reviewed, however, by Tanner,[4] in

[1] Lodder, J., Die Hefesammlung des Centraalbureau voor Schimmelcultures. II Teil. Die anaskosporogenen Hefen, Erste Hälfte. *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II. Deel. **32**: 1–256 (1934).

[2] Copping, A. M., *Biochem. Jour.*, **23**: 1050 (1929).

[3] Williams, R. J., J. L. Wilson, and F. W. von der Ahe, *Jour. Am. Chem. Soc.*, **49**: 227 (1927).

[4] Tanner, F. W., *Chem. Rev.*, **1**: 397 (1925).

1925; by Lutman[1] in 1929; by Buchanan and Fulmer[2] and by Miller[3] in 1930.

Since the appearance of these reviews, considerable new research has been carried out concerning the growth substances of yeasts, and the identities of several components of bios have been established.

In 1935, Thimann[4] outlined the more important advances in the knowledge of bios, while Koser and Saunders[5] (1938) presented an extensive review concerning bacterial accessory growth factors, which included a survey of yeasts and molds.

Origin of the Term.—Wildiers[6] (1901) proposed the name "bios" for the hypothetical organic substance of biological origin that stimulated the growth of yeasts. He based his evidence for the existence of bios on a series of observations made during his researches. He observed that a beer yeast, a strain of *Saccharomyces cerevisiae*, grew poorly on a synthetic medium that contained ammonia as the source of nitrogen, but that the addition of a small amount of organic material, such as beer wort, caused rapid growth of the yeast. Likewise small amounts of inoculum sometimes failed to produce growth in a medium, whereas relatively large quantities of inoculum produced rapid growth. The filtrate from boiled yeast produced the same effect as a large inoculum. Liebig's meat extract and other organic substances of biological origin stimulated growth.

Wildiers's ideas on bios were soon challenged, but they served as a basis for subsequent important research.

Chemistry of Bios.—The complex known as bios is soluble in water and in 80 per cent alcohol but insoluble in absolute alcohol and ether. It is relatively stable to heat and moderately so to acids but is destroyed by boiling in a 20 per cent solution of sulphuric acid. A boiling solution of sodium hydroxide, of concentration much in excess of 1 per cent, usually destroys bios. It is dialyzable through a semipermeable membrane.[7]

Lucas[8] demonstrated that bios may be separated into two fractions

[1] Lutman, B. F., "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929.

[2] Buchanan, R. E., and E. I. Fulmer, "Physiology and Biochemistry of Bacteria," Vol. II, The Williams & Wilkins Company, Baltimore, 1930.

[3] Miller, W. L., *Jour. Chem. Education*, **7**: 263 (1930).

[4] Thimann, K. V., *Ann. Rev. Biochem.*, **4**: 545 (1935).

[5] Koser, S. A., and F. Saunders, *Bact. Rev.*, **2**: 122 (1938).

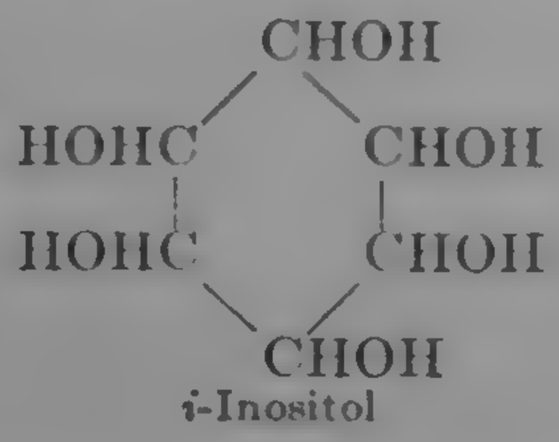
[6] Wildiers, E., *Cellule*, **18**: 313 (1901).

[7] Anderson, C. G., "An Introduction to Bacteriological Chemistry," The Williams & Wilkins Company, Baltimore, 1938.

[8] Lucas, G. H. W., *Jour. Phys. Chem.*, **28**: 1180 (1924).

by the use of an alcoholic solution of barium hydroxide. The barium salt of one fraction, bios I, was insoluble in alcohol, while no salt was formed by the second fraction, which was designated as bios II. Neither of the fractions by itself had much activity, but a combination of the two fractions produced growth stimulation.

1. *Bios I. i-Inositol.*—Eastcott[1] identified bios I as optically inactive inositol, which has the following structural formula:



i-Inositol

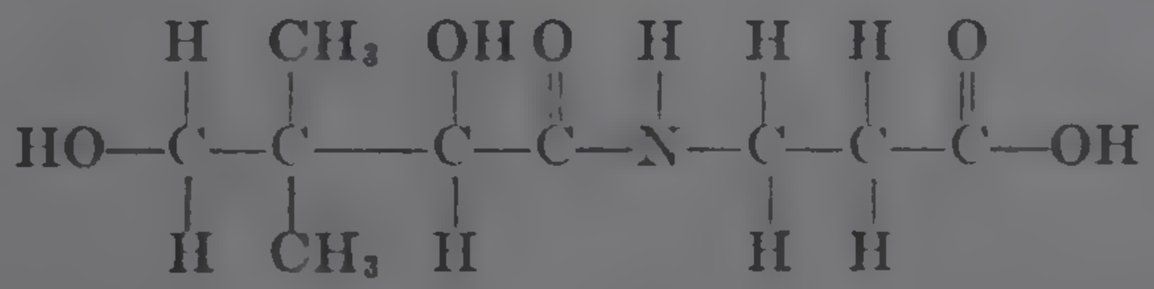
Inositol had very little action when used alone but was effective when combined with other components of bios.

2. *Bios II.*—Bios II, the portion of bios not precipitated by an alcoholic solution of barium hydroxide, has been fractionated into other substances by different methods.

a. PANTOTHENIC ACID.—Using fuller's earth, Williams and his coworkers[2] separated bios II into an adsorbed fraction, replaceable by vitamin B_1 (thiamin or aneurin), and an unadsorbed fraction designated by them as "pantothenic acid."

The term "pantothenic" is derived from Greek and signifies "from everywhere." Pantothenic acid is thus named because it may be isolated from a large number of sources, which include plant (bacteria, slime molds, etc.) and animal tissues.

Pantothenic acid has been synthesized by Stiller, Keresztesy, and Finklestein[3] of the Merck Research Laboratories. It has the following chemical structure:



It is prepared by condensing alpha-hydroxy-beta, beta-dimethyl-gamma-butyrolactone, a product that has been synthesized, with beta-alanine.

Small amounts of thiamin, or relatively large quantities of *i*-inositol,

[1] EASTCOTT, E. V., *Jour. Phys. Chem.*, **32**: 1094 (1928).

[2] WILLIAMS, R. J., and E. BRADWAY, *Jour. Am. Chem. Soc.*, **53**: 783 (1931); and HONN, *Jour. Plant Physiol.*, **7**: 629 (1932).

[3] WILLIAMS, R. J., and R. T. MAJOR, *Science*, **91**: 246 (1940).

or mixtures of the two, increased the stimulating effect of this acid in respect to certain yeasts.[1]

b BIOTIN.—If bios is fractionated with charcoal, the adsorbed portion contains a substance designated by Kögl and his associates[2] as "biotin." This substance may be eluted from charcoal by the aid of an aqueous solution of ammonia and acetone. Afterward the product may be purified. Kögl[3] states that approximately 300 tons of ordinary yeast would be necessary for the production of 1 g. of biotin. The unadsorbed portion has been called bios III.

Biotin has been assigned the tentative formula $C_{11}H_{18}O_3N_2S$. It has been obtained as the methyl ester in crystalline form. One part of biotin in 4×10^{11} parts of medium caused a distinct stimulation, while one part in 10^{9} parts produced a perceptible effect on the growth of yeast strain *M*. The growth effect is increased by the presence of the cofactors inositol and thiamin.

c BETA-ALANINE.—Miller and his associates[4] have separated bios II into two fractions by the use of charcoal. The portion adsorbed by the charcoal and removed by shaking with an aqueous solution of acetone and ammonia was designated as "bios II*B*," while the unadsorbed portion of bios II, the filtrate, was called "bios II.1." The properties of bios II.1 are due to beta-alanine and inositol.[5]

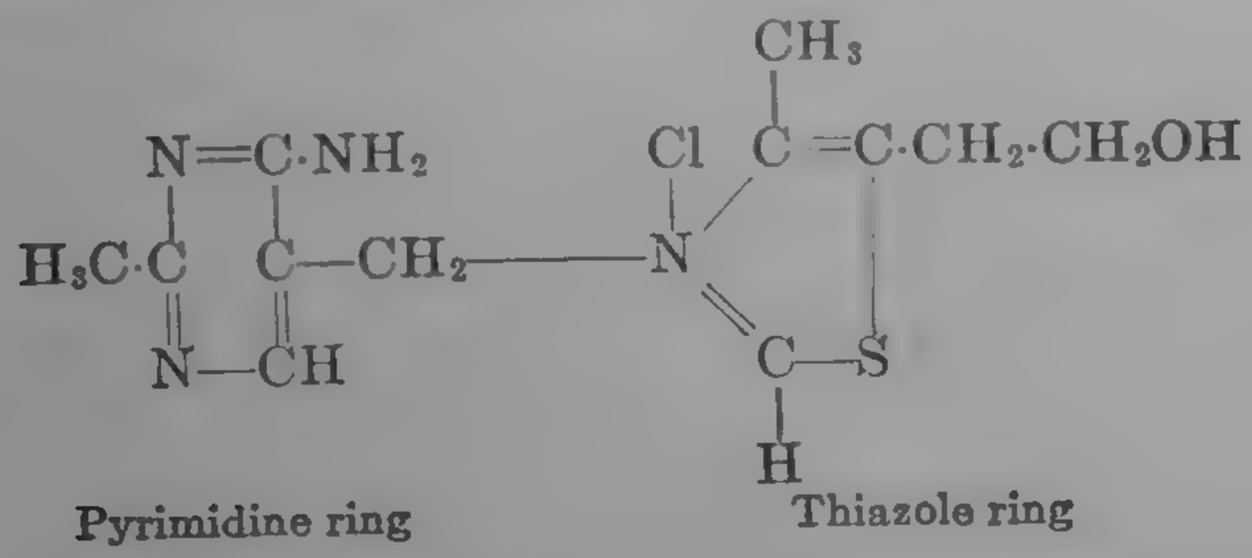
In a medium containing sugar, salts, and 5 mg. of inositol per liter, the growth of several strains of yeasts was stimulated by the addition of as little as 0.08 microgram of beta-alanine per cubic centimeter of medium (approximately 1 part in 12,000,000). Aspartic acid enhanced the effect.[6] Pantothenic acid and thiamin in at least one case, stimulated growth when added.

At very high dilutions, beta-alanine may be slightly inhibitory.[7] Nielsen and Hartelius state that the beta-alanine is toxic to yeast except when asparagine or aspartic acid is a constituent of the medium.[8]

d THIAMIN.—Williams and his associates (1930) observed that "thiamin vitamin B_1 or aneurin" stimulated the growth of a certain strain of yeast.[9]

[1] Williams, R. J., and D. H. Spencer, *Biochem. Jour.*, **28**: 1887 (1934).
[2] Kögl, F., and B. Tonnis, *Zeit. physiol. Chem.*, **242**: 43 (1936); *Chem. & Ind.*, **57**: 113 (1938).
[3] *Ibid.*
[4] Miller, W. L., J. V. Eastcott, and E. M. Stanbury, *Trans. Roy. Soc. Can.* III, **29**: 183 (1935).
[5] Miller, W. L., *Trans. Roy. Soc. Can.*, III, **30**: 99 (1936).
[6] Williams, R. J., and E. Rohrman, *Jour. Am. Chem. Soc.*, **58**: 695 (1936).
[7] *Ibid.*
[8] Nielsen, N., and V. Hartelius, *Biochem. Zeit.*, **296**: 359 (1938).
[9] Williams, R. J., and R. R. Roehm, *Jour. Biol. Chem.*, **87**: 581 (1930).

Thiamin has the following structural formula:



Farrell[1] found that *Saccharomyces hanseniospora valbyensis* reproduced well in a medium containing tomato juice but not in a medium containing inositol, bios II*A*, and bios II*B*. She designated the substance that stimulated growth as "bios V". It has been shown that bios V may be replaced by thiamin.[2]

Thiamin has a marked effect on yeast fermentation, according to Schultz and his coworkers.[3]

Thiamin, derivatives of thiamin, and the pyrimidine and/or the thiazole portions of the thiamin molecule may serve as accessory growth factors for certain microorganisms.[4]

Rhodotorula rubra and *R. flava*[5] are stimulated not only by the intact thiamin molecule but also by the pyrimidine ring of the molecule. A concentration of approximately 0.4 microgram of thiamin in 25 cc. of medium produced maximum growth.

The action of thiamin, pyrimidine, and thiazole on various yeasts has been investigated by Schultz and his associates.[6]

e. BIOS VII.—A "useful constituent," which accompanies bios II*B*, has been named "bios VII" by Miller.[2] Marchant (1942) has shown that bios VII may be almost replaced by pyridoxine.

Sources of Bios Constituents.—Constituents of bios may be isolated from a large number of sources: from wild yeasts, from *Aspergillus niger*,[7] from the leaves and buds of the birch,[8] from malt rootlets,[9] from beer wort, from tomato juice, from the charcoal used in the refining of raw brown sugar, and from many other sources.

[1] FARRELL, L. N., *Trans. Roy. Soc. Can.*, III, **29**: 167 (1935).
[2] MILLER, W. L., *Trans. Roy. Soc. Can.*, III, **31**: 159 (1937).
[3] SCHULTZ, A. S., L. ATKIN, and C. N. FREY, *Jour. Am. Chem. Soc.*, **59**: 948 (1937).
[4] KOSER, S. A., and F. SAUNDERS, *Bact. Rev.*, **2**: 99 (1938).
[5] SCHOPFER, W. H., *Compt. rend.*, **205**: 445 (1937); *Compt. rend. soc. biol.*, **126**: 812 (1937).
[6] SCHULTZ, A. S., L. ATKIN, and C. N. FREY, *Jour. Am. Chem. Soc.*, **60**: 490 (1938).
[7] NIELSEN, N., and V. HARTELIUS, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**: 1 (1937).
[8] DAGYS, J., *Protoplasma*, **24**: 14 (1935).
[9] DEAS, J., *Jour. Biol. Chem.*, **61**: 5 (1924).

Function of Growth Substances.—Thiamin is closely related to cocarboxylase and is believed to function in the structure of the latter compound.

The exact functions of biotin, inositol, β-alanine, and pantothenic acid are not known at present.

For further information concerning this subject, the reader is referred to the publications listed in the following bibliography, some of which contain additional references.

References Concerning the Growth Substances of Yeast

Buchanan, R. E., and E. I. Fulmer: "Physiology and Biochemistry of Bacteria," Vol. II, Williams & Wilkins, Baltimore, 1930.

Copping, A. M.: The Effect of "Bios" on the Growth and Metabolism of Certain Yeasts, *Biochem. Jour.*, **23**: 1050 (1929).

Dagys, J.: Die Hefewuchsstoffe in Knospen und Blättern, *Protoplasma* **26**: 20 (1936).

Devloo, R.: Purification du bios de Wildiers, *Cellule*, **23**: 361 (1906).

Eastcott, E. V.: Wildiers' Bios. The isolation and identification of "Bios I," *Jour. Phys. Chem.*, **32**: 1094 (1928).

Euler, H. von, und T. Philipson: Wasserlösliche Wachstumfaktoren, *Biochem. Zeit.*, **245**: 418 (1932).

Farrell, L. N.: The Influence of Inositol, Bios II*A* and Bios II*B* on the Reproduction of Twelve Species of Yeast. A New Constituent of Bios. *Trans. Roy. Soc. Can.*, III, **29**: 167 (1935).

Fulmer, E. I., W. W. Duecker, and V. E. Nelson: The Multiple Nature of Bios, *Jour. Am. Chem. Soc.*, **46**: 723 (1924).

Hall, H. H., L. H. James, and L. S. Stuart: Yeast Growth Stimulants in White Sugars, *Ind. Eng. Chem.*, **25**: 1052 (1933).

Kögl, F.: On Plant Growth Hormones, *Chem. and Ind.*, **57**: 49 (1938).

——— und B. Tönnis: Über das Bios-Problem. Darstellung von krystallisierten Biotin aus Eigelb. *Zeit. physiol. Chem.*, **242**: 43 (1936).

Koser, S. A., and F. Saunders: Accessory Growth Factors for Bacteria and Related Microorganisms, *Bact. Rev.*, **2**: 99–160 (1938).

Lucas, G. H. W.: The Fractionation of Bios and Comparison of Bios with Vitamins B and C, *Jour. Phys. Chem.*, **28**: 1180 (1924).

Lutman, B. F.: "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929.

Marchant, C.: The Identification of Bios V as Vitamin B_1 and of a Constituent of Bios VII Solution as Vitamin B_6; Their Effect upon the Reduction of *Saccharomyces hanseniaspora valbyensis*, Yeast 2335, and *Saccharomyces galactosus*, *Can. Jour. Research*, **20**: 21–31 (1942).

Miller, W. L.: Wildiers' Bios, *Trans. Roy. Soc. Can.*, III, **29**: 163 (1935).

———: Wildiers' Bios, *Trans. Roy. Soc. Can.*, III, **30**: 99 (1936).

———: Wildiers' Bios, *Trans. Roy. Soc. Can.*, III, **31**: 159 (1937).

———, E. V. Eastcott, and E. M. Sparling: The fractionation of "Bios II," *Trans. Roy. Soc. Can.*, III, **26**: 165 (1932).

Nielsen, N., and V. Hartelius: The Separation of Growth Promoting Substances, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **19**: (No. 8), 1932.

——— und ———: Über die Trennung der auf die Stoffproduktion der Hefe und Schimmelpilzen einwirkenden Wuchsstoffe, *Compt. rend. trav. lab. Carlsberg, Sér physiol.*, **22** (No. 1), 1937.

——— und ———: Wuchsstoffwirkung der Aminosäuren, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22** (No. 14): 249 (1938).

——— und ———: Untersuchungen über die Wuchsstoffwirkung von β-Alanin, β-Alanyl-Glycin, Asparaginsäure, Glycyl-Asparaginsäure und verwandte Stoffe auf Hefe, *Biochem. Zeit.*, **296**: 359 (1938).

RICHARDS, O. W.: The Stimulation of Yeast Growth by Thallium, a "Bios" Impurity of Asparagine, *Jour. Biol. Chem.*, **96**: 405–418 (1932).

——— and M. C. TROUTMAN: Spectroscopic Analysis of the Mineral Content of Yeast Grown on Synthetic and Natural Media, *Jour. Bact.*, **39** (No. 6): 739–746 (1940).

SCHADE, A. L.: Growth Substances in Yeast, *Communications on the Science and Practice of Brewing*, No. 4, December, 1938.

SCHOPFER, W. H.: L'aneurine et ses constituants, facteurs de croissance de Mucorinées (Parasitella, Absidia) et de quelques espèces de Rhodotorula, *Compt. rend. soc. biol.*, **126**: 842 (1937).

———: L'action des constituants de l'aneurine sur les levures (Rhodotorula rubra et flava), *Compt. rend. Acad., Sci.*, **205**: 445 (1937).

SCHOPFER, W. H.: "Plants and Vitamins," Chronica Botanica Company, Waltham, Mass., 1943.

SCHULTZ, A. S., L. ATKIN, and C. N. FREY: Thiamine, Pyrimidine and Thiazole as Bios Factors, *Jour. Am. Chem. Soc.*, **60**: 490 (1938).

SPERTI, G. S., J. R. LOOFBOUROW, and C. M. DWYER: Proliferation-promoting Factors from Ultra-violet Injured Cells, *Studies Inst. Divi Thomae*, **I**: 163 (1937).

STANTIAL, H.: The Effect of Inositol, of Bios II, and of Both Together in the Culture Medium, on the Reproduction of Twelve Kinds of Yeast, *Trans. Roy. Soc. Can.*, III, **26**: 163 (1932).

TANNER, F. W.: The "Bios" Question, *Chem. Rev.* **10**: 397 (1925).

THIMANN, K. V.: Growth Substances in Plants, *Ann. Rev. Biochem.*, **4**: 545 (1935).

WILDIERS, E.: Nouvelle substance indespensable au développement de la levure, *Cellule*, **18**: 313 (1901).

WILLIAMS, R. J.: Growth-promoting Nutrilities for Yeasts, *Biol. Revs.*, **16**: 49–80 (1941).

———, C. M. LYMAN, G. H. GOODYEAR, J. H. TRUESDAIL, and D. HOLADAY: "Pantothenic Acid," a Growth Determinant of Universal Biological Occurrence, *Jour. Am. Chem. Soc.*, **55**: 2912 (1933).

——— and R. R. ROEHM: The Effect of Antineuritic Vitamin Preparations on the Growth of Yeasts, *Jour. Biol. Chem.*, **87**: 581 (1930).

——— and E. ROHRMAN, β-Alanine and "Bios," *Jour. Am. Chem. Soc.* **58**: 695 (1936).

——— and D. H. SAUNDERS: The Effects of Inositol, Crystalline Vitamin B_1 and "Pantothenic Acid" on the Growth of Different Strains of Yeast, *Biochem. Jour.*, **28**: 1887 (1934).

———, J. L. WILSON, and F. H. VON DER AHE: The Control of "Bios" Testing and the Concentration of a Bios, *Jour. Am. Chem. Soc.*, **49**: 227 (1927).

Some General References Concerned with Yeasts

BALTATU, E.: Mycoderma als echte *Saccharomycetes*, *Zentr. Bakt. Parasitenk.*, *Abt.* **II. 101**: 196–225 (1939).

BAUMANN, C. A., and F. J. STARE: Coenzymes, *Physiol. Rev.*, **19**: 353 (1939).

BEDFORD, C. L.: Genus *Hansenula* Sydow. Morphological and Physiological Studies, Doctor's Thesis, University of California, Berkeley (1941).

———: A Taxonomic Study of the Genus *Hansenula*, *Mycologia*, **34**: 628–649 (1942).

BUCHANAN, E. D., and R. E. BUCHANAN "Bacteriology," 4th ed., The Macmillan Company, New York, 1939.

BURK, D., and C. K. HORNER: Nitrogen Assimilation by Yeast, a Consideration of Yeast Nutrition and Metabolism, *Wallerstein Labs. Communs.* **2** (No. 6): 5–23 (1939).

CUSTERS, M. TH. J.: Onderzoekingen over het gistgeslacht *Brettanomyces*, Thesis, *Tech. Hoogeschool*, Delft (1940). Translated by H. J. Phaff and H. C. Douglas, University of California, Berkeley.

DICKENS, F.: Yeast Fermentation, *Biochem. Jour.*, **32:** 1615, 1626, 1645 (1938).

DIDDENS, H. A., und J. LODDER: Die anaskosporogenen Hefen, Zweite Hälfte, N. V. Noord-Hollandsche Uitgevers Maatschappij, Amsterdam, 1942.

ELVEHJEM, C. A.: The Rôle of Iron and Copper in the Growth and Metabolism of Yeast, *Jour. Biol. Chem.*, **90:** 111–132 (1931).

FISCHER, A. M.: Yeast and Factors Determining Its Vitamin Potency, *Brewers Digest*, **13:** 37 (No. 10) (1938).

FRED, E. B., and S. A. WAKSMAN: "Laboratory Manual of General Microbiology," McGraw-Hill Book Company, Inc., New York, 1928.

FREY, CHARLES N.: History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.*, **22:** 1154 (1930).

———, G. W. Kirby, and A. SCHULTZ: Yeast: Physiology, Manufacture and Uses, *Ind. Eng. Chem.*, **28:** 879–884 (1936).

———, A. S. SCHULTZ, and L. ATKIN: Factors Affecting the Fermentation Rate of Yeast, p. 332, Abstracts of Communications, Third International Congress of Microbiology, New York, Sept. 2–9, 1939.

GLAUBITZ, M.: "Atlas der Gärungsorganismen," Paul Parey, Berlin, 1932.

GOTTSCHALK, A.: The Mechanism of Selective Fermentation of *d*-Fructose from Invert Sugar by Sauternes Yeast, *Biochem. Jour.*, **40** (No. 4): 621–626 (1946).

GRAHAM, V. R., and E. G. HASTINGS: Studies on Film-forming Yeasts, *Can. Research*, **19:** 251–256 (1941).

GUILLIERMOND, A.: "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

———: "Clef dichotomique pour la détermination des levures," Librairie Le François, Paris, 1928.

———: "La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents," Masson et Cie, Paris, 1937.

——— et R. GAUTHERET: Action des bleus de Nil et de crésyl sur les levures: réduction et excrétion de ces colorants par les levures, *Compt. rend. Acad. Sci.*, **206:** 1848 (1938).

HENRICI, A. T.: "Molds, Yeasts and Actinomycetes," John Wiley & Sons, Inc., New York, 1930.

———: The Yeasts: Genetics, Cytology, Variation, Classification and Identification, *Bact. Revs.*, **5:** 97–179 (1941).

HOPKINS, R. H., and R. H. ROBERTS: The Kinetics of Alcoholic Fermentation of Sugars by Brewer's Yeast. I. Effect of Concentrations of Yeast and Sugar. *Biochem. Jour.*, **29:** 919 (1935); II. The Relative Rates of Fermentation of Glucose and Fructose, *Biochem. Jour.*, **29:** 931 (1935); III. The Temperature Coefficients of the Rates of Fermentation of Glucose and Fructose, *Biochem. Jour.*, **29:** 2486 (1935); IV. Specificity. The Rates of Fermentation of α- and β-Glucose, *Biochem. Jour.*, **30:** 76 (1936).

JÖRGENSEN, A.: "Practical Management of Pure Yeast," 3d ed., revised by A. Hansen, Charles Griffin & Company, Ltd., London, 1936.

JOSLYN, M. A.: The Mineral Metabolism of Yeast, *Wallerstein Labs. Communs.*, **4** (No. 11): 49–65 (1941).

KLUYVER, A. J., and M. TH. J. CUSTERS: The Suitability of Disaccharides as Respiration and Assimilation Substrates for Yeasts Which Do Not Ferment These Sugars, *Antonie van Leeuwenhoek*, **6**: 121–162 (1939, 1940).

KÖVESSI, F.: Investigations on the Development of Yeast Cells (*Saccharomyces sp.*), Mechanical Equations Expressing the Development of Living Beings, and the Biophysical-Chemical Relationships, p. 336, Abstracts and Communications, Third International Congress of Microbiology, New York, Sept. 2–9, 1939.

KRUIS, K., a J. ŠATAVA: "O vývoji a klíčení spór jakož i sexualitě kvasinek," V Praze, 1918.

KUFFERATH, M. H.: How to Identify Yeasts, II. Asporogenous Yeasts, *Rev. fermentations et inds. aliment.*, **1** (No. 4): 164 (1946).

LANDIS, Q., and C. N. FREY: The Enzymes of Flour in Relation to Yeast Fermentation, *Cereal Chem.*, **13**: 281 (1936).

LANGERON, M., and P. GUERRA: Nouvelles recherches de zymologie, médicale, *Ann. parasitol. humaine et comparée*, **16**: 36–84, 162–179, 429–476, 481–525 (1938).

LAUFER, S., and R. SCHWARZ: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936.

LINDEGREN, C. C., and G. LINDEGREN: Sporulation in *Saccharomyces cerevisiae*, *Botan. Gaz.* **105** (No. 3): 304–316 (1944).

LODDER, J.: Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Konink. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II, **32**: 1–256 (1934).

LUTMAN, B. F.: "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929.

MACFARLANE, M G.: Phosphorylation in Living Yeast, *Biochem. Jour.*, **30**: 1369. (1936).

MCANALLY, R. A., and I. SMEDLEY-MACLEAN: The Synthesis of Reserve Carbohydrate by Yeast. I. Synthesis from Glucose and Maltose and the Influence of Phosphate Thereon, *Biochem. Jour.*, **29**: 1872 (1935).

MCKATER, J. A.: Cytology of *Saccharomyces cerevisiae* with especial reference to nuclear division, *Biol. Bull. Marine Biol. Lab.*, **49**: 213 (1927).

MRAK, E. M., and L. S. MCCLUNG: Yeasts Occurring on Grapes and in Grape Products in California, *Jour. Bact.*, **40**: 395–407 (1940).

———, H. J. PHAFF, and H. C. DOUGLAS: A Sporulation Stock Medium for Yeasts and Other Fungi, *Science*, **96** (No. 2497): 432 (1942).

———, ———, and R. H. VAUGHN: Yeasts Occurring on Dates, *Jour. Bact.*, **43** (No. 6): 689–700 (1942).

———, ———, ———, and H. N. HANSEN: Yeasts Occurring in Souring Figs, *Jour. Bact.*, **44** (No. 4): 441–450 (1942).

NEUBERG, C.: The Biochemistry of Yeasts, *Ann. Rev. Biochem.*, **15**: 435–474, 685–687 (1946).

NICKERSON, W. J.: *Zygosaccharomyces Acidifaciens:* A New Acetifying Yeast, *Mycologia*, **35** (No. 1): 66–78 (1943).

———: Some Trends in Research on Yeasts, *Chronica Botan.*, **7** (No. 8): 409–412 (1943).

———: Studies in the Genus *Zygosaccharomyces* I. Transfer of Pellicle-forming yeasts to *Zygopichia, Farlowia*, **1** (No. 3): 469-481 (1944).

———: Studies on Film-forming Yeasts. Acid Production by *Zygopichia* and *Zygohansenula, Mycologia*, **36** (No. 3): 224–233 (1944).

——— and W. R. CARROLL: On the Metabolism of *Zygosaccharomyces*, *Arch. Biochem.*, **7** (No. 1): 257–271 (1945).

PETT, L. B.: Changes in the Flavine Content of Yeast. *Arkiv Kemi Mineral. Geol.*, **11B:** No. 53 (1935).
———: Studies in Yeast Grown in Cyanide, II, *Biochem. Jour.*, **30:** 1438 (1936).
PYLER, E. J., and T. P. KRUZIC: Yeast Propagation in the Modern Brewery, *Brewers Digest*, **14** (No. 2): 25–7T (1939).
RICHARDS, O. W.: Increased Growth of a Population of Yeast Obtained with Inosite, *Proc. Soc. Expt. Biol. Med.*, **29:** 627 (1932).
———: The Effect of Deuterium on the Growth of Yeast, *Jour. Bact.*, **28:** 289 (1934).
———: Colchicine Stimulation of Yeast Growth Fails to Reveal Mitosis, *Jour. Bact.*, **36:** 187 (1938).
——— and A. J. KAVANAGH: The Course of Population Growth and the Size of Seeding, *Growth*, p. 217, September, 1937.
ROBISON, R.: Chemistry and Metabolism of Compounds of Phosphorus, *Ann. Rev. Biochem.*, **5** (1936).
SALLE, A. J.: "Fundamental Principles of Bacteriology," McGraw-Hill Book Company, Inc., New York, 1948.
ŠATAVA, JAN: "O redukovaných formách kvasinek," V Praze, 1918.
———: "Les formes sexuelles et asexuelles des levures et leur pouvoir fermentatif," IIIe Congrès international technique et chimique des industries agricoles, Paris, 1934.
SCHADE, A. L.: Growth Substances in Yeast, *Wallerstein Labs. Communs.*, **2** (No. 4): 36–40 (1938).
———: The Yeasts, *Wallerstein Labs. Communs.*, **4** (No. 13): 192–193 (1941).
SCHOEN, M.: "The Problem of Fermentation," Chapman Hall, Ltd., London, 1928.
———: Faits nouveaux et nouvelles hypothèses dans la chimie des fermentations (A review), *Ann. fermentations*, **4:** 193–219, 257–283, 321–345 (1938).
SCHULTZ, A. S., and L. ATKIN: The Utility of Bios Response in Yeast Classification and Nomenclature, *Arch. Biochem.*, **14** (No. 3): 36 (1947).
SIZER, I. W.: Sucrose Inversion by Bakers' Yeast as a Function of Temperature, *Jour. Gen. Physiol.*, **21:** 695 (1938).
SKINNER, C. E.: The Yeast-like Fungi: *Candida* and *Brettanomyces*, *Bact. Revs.*, **11** (No. 4): 227–274 (1947). (Article contains 303 references.)
——— and R. BOUTHILET: Melibiose Broth for Classifying Yeasts, *Jour. Bact.* **53** (No. 1): 37 (1947).
SMYTHE, C. V.: Phosphoric acid esters from yeast extract, *Jour. Biol. Chem.*, **117:** 135 (1937).
———: The utilization of pyruvic acid by bakers' yeast, *Jour. Biol. Chem.*, **125:** 635 (1938).
———: The effect of certain tissue extracts, of ammonium salts and of certain amides on the rate of fermentation by bakers' yeast, *Enzymologia*, **6:** 9 (1939).
STANTIAL, H.: The Sporulation of Yeast, *Trans. Roy. Soc. Can.*, III, **29:** 175–188 (1935).
STELLING-DEKKER, N. M.: Die Sporogenen Hefen, *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II, **28:** 1–547 (1931).
STEPHANY, C. D., and H. W. VON LOESECKE: "Selected References on Yeast," War Food Administration, Office of Marketing Services, Washington, D. C., January 1945.
TANNER, F. W., E. D. DEVEREUX, and F. M. HIGGINS: The Multiplication of Yeasts and Yeast-like Fungi Synthetic Nutrient Solutions, *Jour. Bact.*, **11:** 45 (1926).
——— and L. MILLHOUSE: Observations on the growth of yeasts in pure nutrient solutions, *Proc. Soc. Expt. Biol. Med.*, **23:** 447 (1926).

——— and L. B. Strauch: Effect of sodium benzoate upon certain yeasts, *Proc. Soc. Expt. Biol. Med.*, **23**: 449 (1926).

——— and B. W. Williamson: The Effect of Freezing on Yeasts. *Proc. Soc. Expt. Biol. Med.*, **25**: 377 (1928).

Tauber, H.: "Enzyme Chemistry," John Wiley & Sons, Inc., New York, 1937.

Thorne, R. S. W.: The Nitrogen Nutrition of Yeast, *Wallerstein Labs. Communs.*, **9** (No. 27) 97–114 (1946).

Wallerstein, J. S.: New Facts About Growth Substances, *Wallerstein Labs. Communs.*, **4** (No. 11): 14–19 (1941).

———and A. L. Schade: Some Considerations on the Nature of Yeast I. The Structure and Functions of the Cell, *Wallerstein Labs. Communs.*, **3** (No. 9): 91–106 (1940). II. Their Origin and Relation to Other Organisms, *Wallerstein Labs. Communs.*, **3** (No. 10): 182–198 (1940).

Wickerham, L. J.: A Critical Evaluation of the Nitrogen Assimilation Tests Commonly Used in the Classification of Yeasts, *Jour. Bact.*, **52** (No. 3): 293–301 (1946).

———: A Simple Technique for the Detection of Melibiose-fermenting Yeast, *Jour. Bact.*, **46** (No. 6): 501–505 (1943).

——— and A. A. Andreasen: The Lyophil Process. Its Use in the Preservation of Yeast, *Wallerstein Labs. Communs.*, **5** (No. 16): 165–168 (1942).

——— and E. Duprat: A Remarkable Fission Yeast, *Schizosaccharomyces versatilis* Nov. Sp., *Jour. Bact.*, **50** (No. 5): 597–607 (1945).

——— and M. H. Flickinger: Viability of Yeasts Preserved Two Years by The Lyophil Process, *Brewers Digest*, **21** (No. 4) (April, 1946).

———, ——— and K. A. Burton: A Modification of Henrici's Vegetable-juice Sporulation Medium for Yeasts, *Jour. Bact.*, **52** (No. 5): 611–612 (1946).

——— L. B. Lockwood, O. G. Pettijohn, and G. E. Ward: Starch Hydrolysis and Fermentation by the Yeast *Endomycopsis fibuliger*, *Jour. Bact.*, **48** (No. 4): 413–427 (1944).

Winge, Ö.: On haplophase and diplophase in some Saccharomycetes, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **21** (No. 4): 77 (1935).

———and O. Laustsen: On two types of spore germination, and on genetic segregations in Saccharomyces, demonstrated through single-spore cultures. *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22** (No. 6): 99 (1937).

——— and ———: Artificial species hybridization in yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22** (No. 13): 235 (1938).

Some References Concerning Yeast Genetics

Garlan, P.: Fundamentals of Genetics, *Wallerstein Labs. Communs.*, **9** (No. 26): 5–27 (1946).

Henrici, A. T.: The Yeasts, Genetics, Cytology, Variation, Classification and Identification, *Bact. Revs.*, **5**: 97–179 (1941).

Kruis, K., and J. Šatava: O vývoji a klíčení spor jakož; sexua litě kvasinek, V. Praze (1918).

Lindegren, C. C.: The Improvement of Industrial Yeasts by Selection and Hybridization, *Wallerstein Labs. Communs.*, **7** (No. 22): 153–168 (1944).

———: Yeast Genetics, Life Cycles, Cytology, Hybridization, Vitamin Synthesis, and Adaptive Enzymes, *Bact. Revs.*, **9**: 111–170 (1945).

———: Breeding Yeasts for Their New Role in Nutrition, *Missouri Botan. Garden Bull.*, **34**: 37–43 (1946).

——— and G. Lindegren: Vitamin-synthesizing Deficiencies in Yeasts Supplied by Hybridization, *Science*, **102** (No. 2637): 33–34 (1945).

———, S. Spiegelman, and G. Lindegren, Mendelian Inheritance of Adaptive Enzymes in Yeast, *Proc. Nat. Acad. Sci.*, **30**: 346–352 (1944).

Šatava, J.: Oredukovaných formách krasinek V. Praze (1918).

———: Les formes sexuelles et asexuelles des levures at leur pouvoir fermentatif—III^{e} Congrès international technique et chimique des industries agricoles, Paris (1918).

Spiegelman, S., and C. C. Lindegren: The Relation of Sporulation and the Range of Variation of the Haplophase to Population Adaptation, *Jour. Bact.*, **49**: 257–269 (1945).

———, ——— and G. Lindegren: Maintenance and Increase of a Genetic Character by a Substrate–Cytoplasmic Interaction in the Absence of the Specific Gene, *Proc. Nat. Acad. Sci.*, **31**: 95–102 (1945).

Winge, Ö.: On Haplophase and Diplophase in Some Saccharomycetes, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **21** (No. 4): 77–111 (1935).

——— and O. Laustsen: On Two Types of Spore Germination, and on Genetic Segregations in *Saccharomyces*, Demonstrated through Single-spore Cultures, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*), **22** (No. 6): 99–116 (1937).

——— and ———: Artificial Species Hybridization in Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol*, **22** (No. 13): 235–244 (1938).

——— and ———: On 14 New Yeast Types, Produced by Hybridization, *Compt. rend. trav. lab. Carlsberg, Sér. physiol*, **22**, 337–352 (1939).

——— and ———: *Saccharomycodes ludwigii* Hansen, A Balanced Heterozyzote, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**: 357–370 (1939).

——— and ———: On a Cytoplasmic Effect of Inbreeding in Homozygous Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **23**: 17–39 (1940).

CHAPTER III

SACCHARIFYING AGENTS: METHODS OF PRODUCTION AND USES

Introduction.—Starches, hemicelluloses, and celluloses must be hydrolyzed or converted to fermentable sugars by enzymatic or chemical agencies before they may be used for certain industrial purposes, such as ethanol production. A variety of methods may be used for converting complex carbohydrates to relatively simple materials. Some of these methods are applicable in the case of starches alone and others, with suitable modifications, may be used for the saccharification of either starches or cellulose materials.

Methods of Saccharifying Starch-containing Materials.—In general, the methods of saccharifying starch-containing materials involve the use of enzyme preparations or dilute acids, or a combination of the two. The enzyme preparations that may be used include malt, which is of cereal origin, and those of microbial origin, among which are mold and bacterial products. Illustrative of preparations of fungal origin are mold bran and fungal amylases. Either liquid or solid bacterial preparations are available. A method that depends upon growing an amylase-producing mold in the substrate that is to be fermented after saccharification is known as the Amylo process. Dilute acids, particularly hydrochloric acid, may be used to convert grains, potatoes, and other starch-containing raw materials. Various combinations of malt with mold bran, or other enzyme preparations of microbial origin, may be employed; or combinations of acid with mold enzymes may be used. The method of conversion selected will depend, of course, on the use intended and the availability of the hydrolytic agents and their relative costs.

Cooking the Mash.—The purpose of cooking grain mashes is to prepare them for the conversion process in which starches are saccharified to fermentable sugars. During the cooking, the starches are solubilized and gelatinized.

Cooking may be accomplished by batch or continuous processes. In batch operations, cooking may be done at atmospheric pressure with temperatures below the boiling point of water by methods known as infusion processes; or under pressure. The continuous processes utilize high temperatures, obtained by the use of high steam pressures, and short periods of time, usually less than one minute.

Infusion Process.—Some materials, such as wheat, rye, rye malt, and barley malt, may be adequately cooked by infusion processes. For example, wheat may be prepared in accordance with the following method of Stark, Adams, Scalf, and Kolachov.[1] Ground wheat is added slowly to water at a temperature of 100°F., while being agitated continuously. The temperature of the mash is elevated to 155°F. during a period of 45 min., held at 155°F. for 60 min., and then lowered to 152°F. At this temperature a slurry of malt may be added. Conversion may be carried out at 145°F. for 30 min.

Batch-type Pressure Cooking.—This process is carried out in tanks[2] of large capacity, for example, 10,000 gal. or more. The tanks are equipped with agitators, inlets for the water and the grain, outlets, steam spargers, usually a means for producing vacuum, and with other accessories.

During operation, water at a temperature of 120 to 140°F. is added to the tank in an amount sufficient to supply 18 to 22 gal. for each bushel of corn or other grain. Sulphuric acid is added to bring the pH of the mash to 5.4 to 5.6. Then the ground corn is added slowly, with agitation. The mash is heated to 305 to 310°F. by steam under pressure during a period of 45 min. and held for 5 to 10 min. It is cooled to 152°F., first by blowing it down to atmospheric pressure, and second by using vacuum.

The comparatively large floor space occupied by the tank, the relatively long period of processing (3 to 4 hr.), and the difficulty of obtaining uniform mixing and temperature are disadvantages.

Continuous Pressure Cooking.—The most modern method for cooking corn and other grains is a continuous pressure process, which was developed by Unger.[3]

The effects of variables on the efficiency of operation was studied by Unger. The variables were (1) the temperature of cooking, (2) the time of cooking, (3) the particle size of the ground grain, (4) the ratio of the amount of grain to water, (5) the pH of the mash, and (6) agitation. He found that the most important variables were temperature and time and that corn meal may be adequately cooked in 16 to 60 sec. at 350 to 365°F. in a Schutte-Koerting jet heater. He showed that grinding was not too important a factor, provided that the grain was ground enough to be cooked properly. The optimum practical pH range was 5.4 to 5.6.

A continuous cooker system with a capacity for 5,000 bu. of grain per day was designed by Unger and constructed (refer to Fig. 8).

[1] Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov, *Ind. Eng. Chem.*, 15: 443 1943.

[2] Unger, E. D., Thesis, Case School of Applied Science, May, 1941.

[3] *Ibid.*

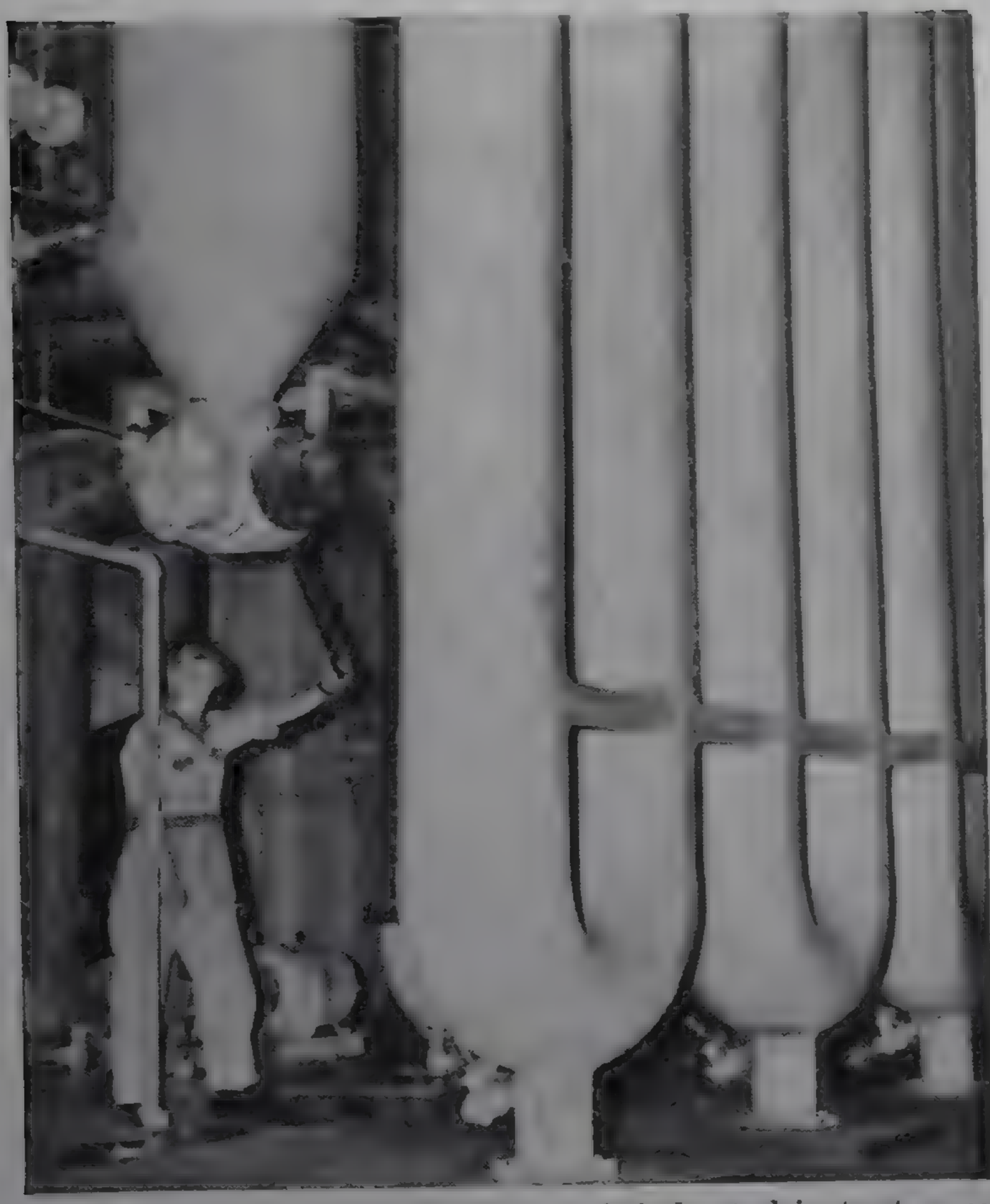


FIG. 8.—Tubes for continuous cooking. The mash is heated instantaneously with steam to 350 to 360°F. by a jet heater. It passes through a series of tubes where it is held for 60 to 70 sec. It is then cooled to 145°F. in a vacuum flash chamber. (*Courtesy of Joseph E. Seagram & Sons, Inc., Louisville, Ky.*)

For further details, the reader is referred to other sections of this book, to "Food for Thought" by Willkie and Kolachov, to Unger's thesis, and to other sources.

MALT

Malt is an enzymic product prepared usually from selected barley, although other cereal grains may be used.

The Preparation of Malt.—The manufacture of malt, commonly carried out by malsters, consists of steeping selected, screened barley, permitting it to germinate, and drying it under carefully regulated conditions. A schematic diagram of a malting plant is shown in Fig. 9.

Selection of Barley.—Barley is selected for the results that it produces. It should be of a good variety, preferably large grained, of a fairly uniform size, and of a light yellow color when matured. It should possess high germinating power, contain but a small amount of bitter and harsh substances in the husk, and have the ability to produce enzymes of the

proper character and quantity during germination. Soft, starchy barley is desirable; damaged or weedy barley is undesirable.

Certain types of barley are sought for the manufacture of fine beer. In the United States malt is prepared principally from six-rowed barley, although some two-rowed barley is used in California and much of it in Europe.

Composition of Barley. Barley contains the four plant proteins: glutelin, hordein, leucosin, and edestin. Glutelin and hordein are found

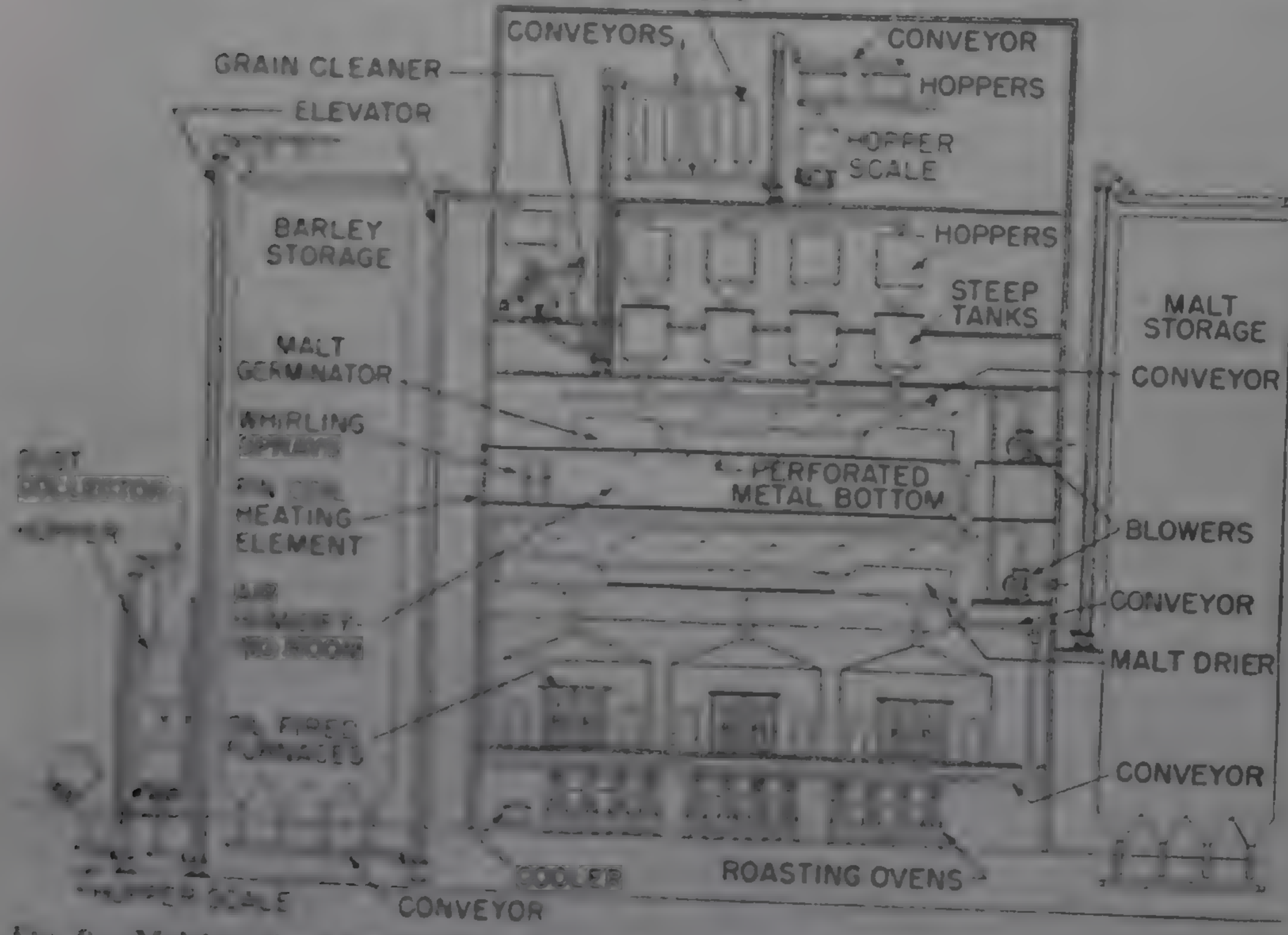


FIG. 9.—Malting plant [illegible] Courtesy of the Editor, [illegible]

[illegible] the husk and aleurone cell layers; leucosin and edestin in the endosperm between the starch granules.

Starch is found in the endosperm and is a most important constituent. Fat, rich in lecithin, is found in the embryo and in the aleurone cells. Enzymes and [illegible] are located principally in the husks. Together with the proteins, the tannins and bitter resins are collectively referred to as "malting aids."

Storing, Cleaning, Grading.—After receipt at the malting plant, the barley is weighed, stored, cleaned, and graded. It may be stored in bins, according to the variety. From the bins, it is conveyed to the cleaners which are of various designs and construction but which generally employ

concentration of water favors normal germination

Water of a known chemical composition and of a definite pH should be used during the steeping process. The composition of the water has much to do with the proper removal of the starch bodies and some proteins, which are undesirable for beers. Some chemicals that tend to make more readily at a higher pH, like sodium carbonate, are sometimes added to the water to obtain grains of a uniform size. The moisture content can then be controlled more readily during steeping.

During steeping, water is imbibed by the individual grains until the moisture content reaches about 45 to 47 per cent. This concentration of water favors normal germination.

Water of a known chemical composition and of a definite pH should be used during the steeping process. The composition of the water has much to do with the proper removal of the starch bodies and some proteins, which are undesirable for beers. Some chemicals that tend to make more readily at a higher pH, like sodium carbonate, are sometimes added to the water to obtain grains of a uniform size. The use of dilute sodium hydroxide solutions in the steep is of advantage when malt, and subsequently beer, is made from grains that have been steeped under acidic conditions. Hypochlorite and permanganate have been used to sterilize the steep water. It is claimed that they also stimulate germination and thereby forestall subsequent infections in the steep. The value of these procedures is not firmly established, however.

The rate at which the water is absorbed by the grains depends on the variety of the barley, the size of the grain, and the temperature of the water.

A temperature of 50° to 60° F during the steeping tanks is satisfactory. Control of the temperature is very important.

Malting is essentially a vital process involving growth and respiration. Respiration rates increase with a rise in temperature and also with increased water content, and oxygen is naturally required. Therefore, during the steep water is removed, and subsequently here, to make room for the air necessary for respiration of the sprouting grains. As soon as the steeping tank is emptied, the grain must be removed by shoveling or by mechanical means, the water must be drained off and the tank refilled.

Understeeping results in a reduced rate of respiration, slowed growth of the rootlet and an incomplete breakdown of the protein. Less extract can be secured from the finished product.

Oversteeping leads to the production of a higher percentage of ungerminated grains, caused by a deficiency in the oxygen supply during steeping, the inadequacy being due to the increased rate of respiration. If the water is adequately aerated, the grains will germinate subsequently, but the development is likely to be abnormal. Hence oversteeping may lead to low yields of malt.

Germination.—During germination several complex changes take place. The visible morphological changes include the formation of the acrospire, or plumule, and rootlets. A histological examination would show the disappearance of the cell walls of the endosperm, while a biochemical analysis would indicate that certain metabolic changes had taken place—the breakdown of proteins, starches, and other complex constituents under the influence of enzymes. Enzymes are elaborated or activated when the temperature, moisture, and aeration conditions are satisfactory for the germination of the seed.

The temperature, the moisture content of the grains, and the oxygen supply are very important during germination. These are controlled carefully in compartments or in revolving drums. Compartments are usually long and narrow. They are equipped with perforated metal bottoms, which allow temperature-controlled, humidified air to pass up through and water to drain out. Conveyors or spouts distribute the steeped barley over the compartment floor. During the germination process, the barley is aerated with humidified air and stirred by special screw propellers which constantly travel back and forth from one end of the compartment to the other, and may be watered by overhead devices. The temperature of the grain is maintained between 60 and 70°F. by controlling the temperature of the incoming air. In rotating drums the humidified and temperature-controlled air enters each drum through a series of inlet tubes located near the periphery and leaves through an outlet near the center. The drums revolve slowly, usually at 1 to 2 r.p.m. during the germination process. Germination generally requires 5 to 7 days. The process is complete when the acrospire has grown to a length equal to three-fourths to the full length of the kernel.

The oxygen supply is especially important during germination, for the rate of respiration increases greatly in germinating grain. During respiration, heat energy is evolved, while carbon dioxide and water are produced as waste respiratory products. The accumulation of carbon dioxide inhibits normal respiration, and its concentration or a deficiency of oxygen, or both, induces abnormal respiration in which hydrogen acceptors other than oxygen may function. The products formed during abnormal respiration may include acids, alcohols, and aldehydes, which exert a toxic effect on the germinating grain or young plant.

Germinating barley usually shows a respiratory coefficient of approximately 1.

$$\text{Respiratory coefficient} = \frac{\text{volume } CO_2 \text{ evolved}}{\text{volume } O_2 \text{ absorbed}}$$

In the case of abnormal respiration, the respiratory coefficient increases, for the carbon dioxide increases, the oxygen decreases, or both changes occur simultaneously. For these reasons it is most important to secure good carbon dioxide removal and to turn and properly aerate germinating barley.

Drying the Germinated Barley.—When the barley has reached the desired stage of growth, drying stops the germination processes and with the application of heat develops color, flavor, and aroma and reduces the moisture content of the malt. A final moisture content of approximately 5 per cent is sought. Malt may then be stored without danger of breakdown by microorganisms.

Drying is carried out in thermostatically controlled kilns or drums at carefully regulated temperatures. The temperatures used have much to do with the enzyme content of the final malt and the flavor of beers made from it. Since much damage can be done to germinated barley by the application of too high temperatures when the grain is wet, low temperatures must be used at first. The temperature is raised gradually, or elevated by small increments, until a final kilning temperature of 75 to 100°C. is used.

The final temperature used depends upon the nature of the malt desired. For a light malt, a lower temperature should be used; for a dark malt, a higher temperature.

During recent years, some malts have been dried in vacuum drums. Such malts possess a high concentration of enzymes but, though satisfactory for use in distilleries, are not so suitable for brewing, since the usual protein changes are affected.

Following the kilning, the malt may be treated, usually by friction, to remove the radicles.

Uses.—Malt has many uses. It is used extensively in the brewing industry as the chief raw material in the manufacture of beers and ales; and as the saccharifying agent in the manufacture of industrial alcohol and distilled liquors from grains, such as wheat, corn, and rye. Malt is also used in the manufacture of malted milk, candies, cereals, and food colorings but is usually roasted first in special ovens under carefully controlled conditions.

Rapid Conversion of Mash.—Gallagher and his associates[1] have described a fast method for converting grain mashes, which was based on

[1] Gallagher, F. H., H. R. Bilford, W. H. Stark, and P. J. Kolachov, *Ind. Eng. Chem.*, **34**: 1395 (1942).

experimental data of the following nature secured in the laboratory. Corn cooks (mashes) were prepared under the same conditions and cooled to 62.8°C., the temperature of conversion. Barley malt (10 per cent), in the form of a slurry, was added in equal amounts to each cook. The mixture of malt and corn was agitated mechanically and conversion was carried out for specified times. The individual mashes were cooled quickly (within 7 to 10 min.) to 22.2°C. and made up to a concentration equivalent to 38 gal. of mash per bushel of grain. The pH was adjusted to 4.8 to 5.0. Then each mash was divided into four portions, three of which were inoculated with *S. cerevisiae* (Seagram No. 1 strain) and one of which was reserved for control purposes. Fermentation was permitted to continue for 68 to 72 hr., after which the mashes were analyzed for residual sugar and alcohol. Data based on conversion periods of 1, 5, 15, 30, and 45 min. are presented in Table 10.

TABLE 10.—COMPARISON OF EFFECT OF MASH CONVERSION TIMES ON FERMENTATION YIELDS[1]

Conversion time, min.	Total sugar			Efficiency, per cent		Grams abs. alcohol in 100 cc.
	Initial g./100 ml.	Final g./100 ml.	Fermented, per cent	Fermentation	Plant basis	
1	11.61	0.627	94.5	98.4	93.1	5.52
		0.629	94.5	99.1	93.8	5.56
		0.630	94.5	98.4	93.1	5.52
5	11.58	0.616	94.6	99.3	93.9	5.56
		0.672	94.2	99.4	93.8	5.55
		0.685	94.0	100.7	94.8	5.61
15	11.54	0.745	93.5	100.2	93.5	5.52
		0.715	93.8	101.1	94.7	5.59
		0.752	93.8	100.0	93.4	5.51
30	11.28	0.810	92.8	99.3	92.2	5.31
		0.856	92.4	102.5	94.8	5.46
		0.792	93.1	95.0	88.5	5.10
45	11.87	0.856	92.8	98.0	90.9	5.52
		0.885	92.5	89.1	82.3	5.00

[1] GALLAGHER, F. H., H. R. BILFORD, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1395 (1942).

Additional data obtained by Gallagher, and his coworkers[1] showed that the 1-min. conversion period was equal to or better than the 60-min. period at a pH of 5.4 or 5.9, the lower and upper limits, respectively, of

[1] GALLAGHER, F. H., H. R. BILFORD, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1395 (1942).

levels optimum for plant conversions. More than 70 per cent of the cooked starch in the grain was converted to maltose by barley malt in 1 min. at 62.8°C.

The rapid method for the conversion of mashes is as follows: A proportioning pump continuously injects a slurry of malt into a stream of cooked mash which is being forced along by another pump. The products flow through a pipe 105 ft. long and 4 in. in diameter at a temperature of 62.8°C. The malt acts on the grain for a period of 40 sec. The mixture is then cooled during a period of 1.5 min. to 21.1 to 23.9°C. This particular unit has a capacity for converting 5,000 bu. of grain per day.

THE BALLS-TUCKER PROCESS

This is a process for utilizing the enzymes normally occurring in wheat for saccharification purposes in order to save malt.[1] Wheats contain adequate amounts of beta-amylase but are deficient in alpha-amylase. Accordingly, it is necessary to use some malt to compensate for the paucity in α-amylase.

The process is briefly as follows: A slurry of ground wheat is acidified to a pH of 5.2 to 5.8 and 0.05 to 0.1 per cent of Na_2SO_3 is added to activate the inactive form of amylase and to aid in the flocculation of wheat gluten. The mixture is agitated vigorously (for 1 hr. at 25°C. in one modification) and then permitted to stand for 45 min. or longer. The starch settles out and a considerable portion of the gluten rises to the surface and may be recovered. A portion of the clear supernatant fluid of the middle layer, which contains the activated amylases, is removed and later used to saccharify the cooked mash. Generally about 20 to 35 per cent of the wheat used may be extracted with sulphite. At least 2 per cent of malt on the basis of the weight of the grain is necessary for saccharification. For further details, the reader is referred to the report on "The Production of Ethyl Alcohol from Wheat" by the Northern Regional Research Laboratory, U.S. Department of Agriculture, March, 1944.

MOLD BRAN

Mold bran is an enzyme product obtained by growing *Aspergillus oryzae* on moist, sterilized bran. It is and may be used successfully as a substitute for malt in the saccharification of grain, potato, or other types of starch-containing mashes. Smaller quantities of mold bran than of malt are required to saccharify a given quantity of grain mash. Yields of ethyl alcohol from mashes saccharified with mold bran are, on the average, equal to and often superior to those obtained from mashes saccharified with malt.

[1] BALLS, A. K., and I. W. TUCKER, *Fruit Products Jour.*, **23**: 15–16, 21 (1943).

Development of Process.—Takamine,[1] in 1914, advocated the use of mold enzymes from *A. oryzae* in the distilling industry. Studies were carried out in distilleries in Canada using his mold-bran preparation (*Takadiastase*) in place of malt in saccharifying grains. Although the yields of alcohol obtained through the use of mold bran were reported to be higher than those obtained through the use of the malt, the process was not adopted.

In 1929 Underkofler, Palmer, and Schwarze[2] demonstrated by laboratory methods that higher yields of ethyl alcohol, on the average, could be obtained from corn meshes saccharified by the use of mold bran than from those saccharified by the use of barley malt. This work was later confirmed by Stark and Hoop associates at the Iowa State College[3,4,5,6] and by Roberts, Lauter, Stewart, and Sabetan.[7] Similar findings on a commercial basis have been reported by Boyer and Underkofler;[8] Underkofler, Severson and Goering;[9] and Underkofler, Severson, Goering, and Christensen.[10] The favorable use of mold bran for the conversion of potato meshes was reported by Beresford and Christensen.[11]

Molds Used.—Hao, Palmer, and Underkofler[12] studied mold bran prepared from 27 strains of molds of the genera *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus*. They found that bran preparations made with strains of *A. oryzae*, *R. delemar*, and *R. oryzae* were optimum for the saccharification of corn meshes as evaluated by the yields of ethanol produced. Strains of *A. oryzae* were selected as being best suited for industrial use, based on ease of handling, consistency of results, and high yields of alcohol from the saccharified meshes.

Table II shows the effect of saccharification temperature and time on

[1] Takamine, J., *Ind. Eng. Chem.*, **6**: 824 (1914).

[2] Underkofler, L. A., E. I. Fulmer, and L. Schwarze, *Ind. Eng. Chem.*, **31**: 734 (1939).

[3] Stone, L., E. I. Fulmer, and L. A. Underkofler, *Ind. Eng. Chem.*, **32**: 544 (1940).

[4] Underkofler, L. A., *Brewers Digest*, **17** (No. 11): 29 (1942).

[5] Hao, L. C., E. I. Fulmer, and L. A. Underkofler, *Ind. Eng. Chem.*, **35**: 81 (1943).

[6] Underkofler, L. A., and E. I. Fulmer, *Cereal Chem.*, **7**: 120 (1943).

[7] Roberts, R. W., and L. A. Underkofler, *Cereal & Wet Proc.*, December, 1945.

[8] Underkofler, L. A., G. M. Severson, and K. J. Goering, *Ind. Eng. Chem.*, **38**: (1946).

[9] Underkofler, L. A., G. M. Severson, K. J. Goering, and L. M. Christensen, *Ind. Eng. Chem.*, **38**: (1946).

[10] Beresford, H., and L. M. Christensen, Bul. 241, Wash. Agr. Expt. Sta., September, 1946.

[11] Hao, Fulmer, and Underkofler, *loc. cit.*

alcohol yields from corn mashes saccharified by bran preparations made from selected strains of molds.

Production.—Mold bran may be produced by laboratory, pilot-plant, or commercial methods, some of which are described below.

Laboratory Methods.—The present discussion will be confined to a drum, a pot, and a tray method for preparing mold bran.

DRUM METHOD.—Spore cultures are prepared in flasks to serve as inoculums for the moist, sterilized bran in drums, in accordance with a procedure devised by Underkofler, Severson, Goering, and Christensen.[1]

TABLE 11.—EFFECT OF SACCHARIFICATION TEMPERATURE AND TIME ON ALCOHOL YIELDS FROM CORN MASHES SACCHARIFIED BY SEVERAL MOLD-BRAN PREPARATIONS[1]

Mold used in mold-bran preparation	Saccharification		Mold bran as per cent of corn	Alcohol yield as per cent of theoretical
	Temperature, °C.	Time, hr.		
Aspergillus oryzae (A.T.C.C. No. 4814)*	30	1	4	88.6
	30	1	6	91.5
	30	1	8	91.3
	55	1	4	87.0
	55	1	6	88.4
	55	1	8	89.5
	55	2	4	86.1
	55	2	6	88.0
	55	2	8	89.5
Aspergillus oryzae (Rohm and Haas No. 38)†	30	1	6	94.5
	30	1	8	94.8
	55	3	6	93.5
	55	3	8	93.7
Rhizopus oryzae (Lockwood No. 660)‡	30	1	6	92.0
	30	1	8	93.5
	55	3	6	93.0
	55	3	8	94.0
Rhizopus delemar (NRRL No. 1472)§	30	1	6	92.5
	55	3	6	91.0

[1] HAO, L. C., E. I. FULMER, and L. A. UNDERKOFLER, *Ind. Eng. Chem.*, **35**: 814 (1943).

* American Type Culture Collection, Washington, D. C.

† Rohm and Haas Co., Bristol, Pa.

‡ Dr. L. B. Lockwood, U.S. Department of Agriculture.

§ Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

Ten-gram amounts of the following medium are distributed in 250-ml. flasks and sterilized in the autoclave: 10 g. of ground corn, 100 g. of wheat bran, and 60 ml. of 0.2 N hydrochloric acid containing 0.62 p.p.m. of

[1] UNDERKOFLER, SEVERSON, GOERING, and CHRISTENSEN, *loc. cit.*

$ZnSO_4 \cdot 7H_2O$, 0.63 p.p.m. of $FeSO_4 \cdot 7H_2O$, and 0.08 p.p.m. of $CuSO_4 \cdot 5H_2O$. After cooling, the moist, sterilized bran is inoculated with a culture of *A. oryzae*. The mixture is then distributed on one side of the flask and incubated at 30°C. After sporulation has reached a maximum, rapid drying of the culture is encouraged. The dry spore culture should be greenish in color, according to Underkofler and his associates, and not brown or black. The ratio of the inoculum for moist, sterile bran is about 1 per cent.

The well-sporulated bran culture thus prepared is used to inoculate a much larger charge (1,000 to 1,200 g.) of sterile bran of similar moisture content contained in a drum of 5-gal. capacity and capable of being rotated. During operation, air is passed slowly into the drum, which is maintained at a temperature of about 30°C. preferably (never above 35°C.) to favor the growth of the mold. During the germination period, the drum is rotated for 15 to 20 min. or less each 2 hr. Thereafter, the drum may be rotated continuously at not more than 1 r.p.m. until, at the end of 40 to 54 hr., the moldy bran is removed from the drum. It is then spread thinly on a papered surface and dried at room temperature. Sporulation proceeds during the drying. Before being used, the dried product is ground in the same manner as malt.

POT METHOD.—Hao, Fulmer, and Underkofler[1] developed a laboratory method for the production of mold bran in 1943 which they stated was superior in the following respects to the drum method used earlier: The equipment required less space and was less complicated; the mold mycelium was not disturbed during growth; and uniform aeration was obtained. Furthermore, the molds grew more rapidly in the pots and the resultant mold bran produced relatively consistent and higher yields of ethanol. The method consisted of growing selected strains of molds on wheat bran mash in 3-qt. aluminum pots especially equipped for aeration. The apparatus, a modification of that used by Beresford and Christensen,[2] is shown in Fig. 10.

A detailed description of the pot method follows:

A bran mash is prepared by mixing 750 g. of 0.3 N hydrochloric acid with 750 g. of wheat bran. The moistened bran is packed into the aluminum pot and sterilized with steam at 15 lb. pressure for 30 min. The mash is cooled and then inoculated with 5 to 10 g. of a well-sporulated culture of the mold (usually a strain of *A. oryzae*) which has been grown on wheat bran in flasks as described above. The seeded bran mash is packed firmly into the special pot, the latter being placed on a layer of cotton batting. The pot is incubated at 30°C. but the temperature rises

[1] Hao, Fulmer, and Underkofler, *loc. cit.*

[2] Beresford and Christensen, *loc. cit.*

because of rapid growth of the mold and in about 8 hr. reaches 37 to 40°C. Air is passed through the mass at a pressure equal to 0.3 to 3 in. of water and at such a rate that the temperature is kept below 45°C. Aeration is continued for 12 to 24 hr. longer and then the moldy bran is removed from the pot, spread uniformly on paper, and permitted to dry at room temperature.

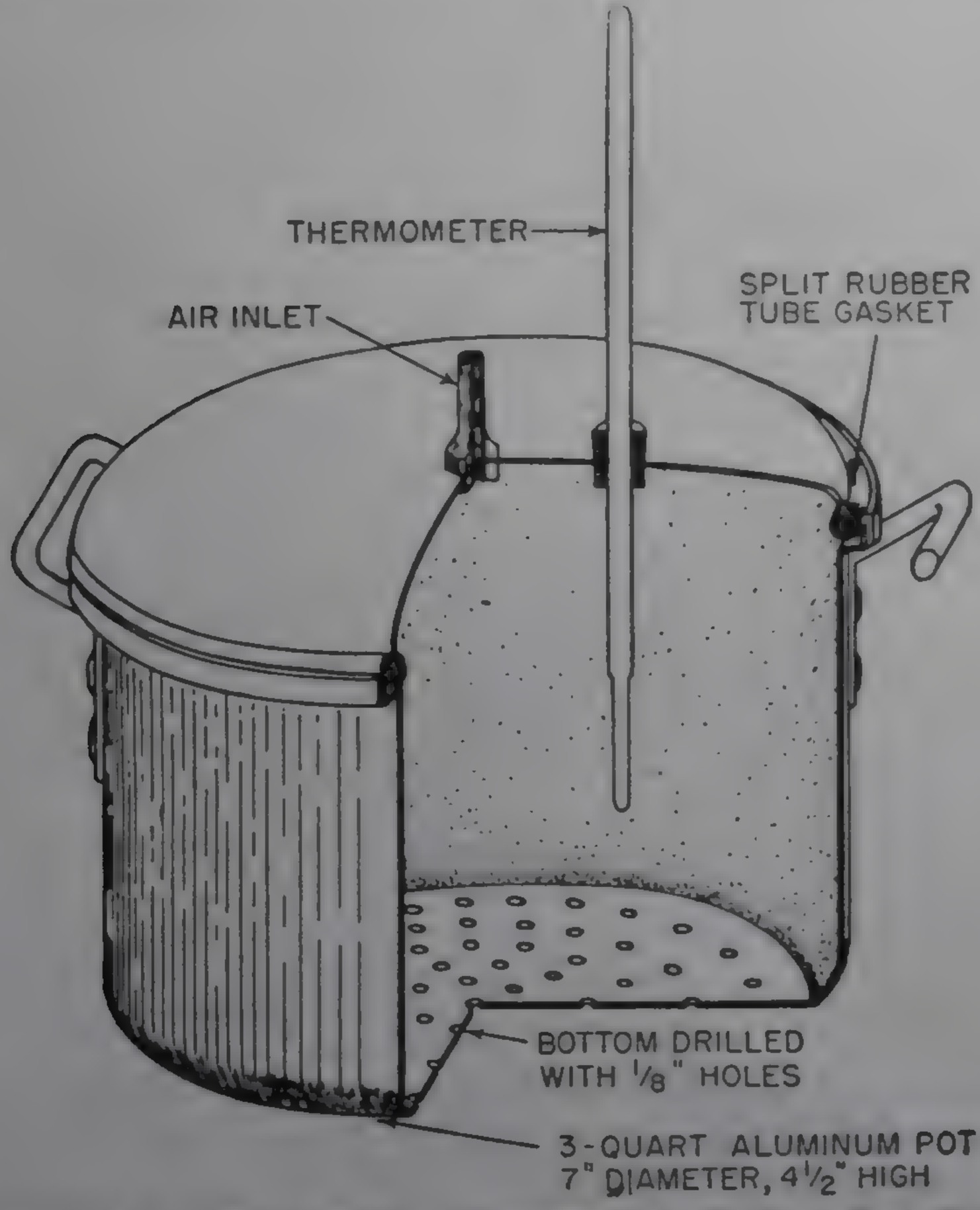


FIG. 10.—Apparatus for growing mold. [*Courtesy of L. C. Hao, E. I. Fulmer, and L. A. Underkofler, Ind. Eng. Chem.*, **35**: 814 (1943).]

TRAY METHOD.—The following method, described by Roberts and his collaborators,[1] is employed for preparing mold bran in trays. Equal weights of wheat bran and water are mixed, and the mixture is spread in layers 1 in. deep in aluminum trays. The moistened bran is sterilized in the trays with steam at a pressure of 20 lb. for 30 min., cooled, and inoculated with a culture of *A. oryzae*, using aseptic technique. The seeded bran is incubated at 25°C. until the mold begins to sporulate, which generally requires about 4 days. The mold spores may be destroyed by adding 95 per cent ethanol to the mold-bran mixture in the proportion of

[1] ROBERTS, LAUFER, STEWART, and SALETAN, *loc. cit.*

a [illegible] of ethanol to [illegible] g. of [illegible] and 500 g. of water. The alcoholic [illegible] is removed while in hydraulic press. The resulting dried, ground press cake is known as "Pelleton-C."

Pilot-plant Methods.—These are concerned principally with problems of engineering laboratory procedures to plant-scale operation and are based on the project by Hinkelbein, Severson, Goering, and Christensen.[1]

FIG. 11.—[illegible] (Courtesy of [illegible] and [illegible] C. M. Severson, K. J. Goering, and L. M. Christensen, Utah [illegible] **24** (No. 1): 1 (1947).]

[illegible] operations.—These operations were accomplished in a special [illegible] (see Fig. 11), which consisted of a covered mixer with [illegible] for [illegible]. The mixer was 18 in. in diameter and 8 in. long. It was [illegible] and when operated at a speed of 23 r.p.m. agitated the [illegible] effectively. A row of jets at the bottom of the cooker admitted steam for sterilization.

During operations, the [illegible] was mixed with an acid-salt solution in the special mixer, "conditioned" by the injection of steam for 30 min., and sterilized continuously. A final moisture content of

[illegible]

about 51 per cent was obtained by Underkofler and his collaborators[1] by adding 1 part of acid (0.2 N HCl) containing double the desired amount of mineral salts to 2 parts of bran. Previous studies had established the fact that practical sterility of the acid-moistened bran could be obtained by heating it at 93 to 99°C. for 15 to 30 min.; that butyric acid bacteria, a bad type of contaminant, could be eliminated; and that the few surviving mold spores caused no serious difficulties.

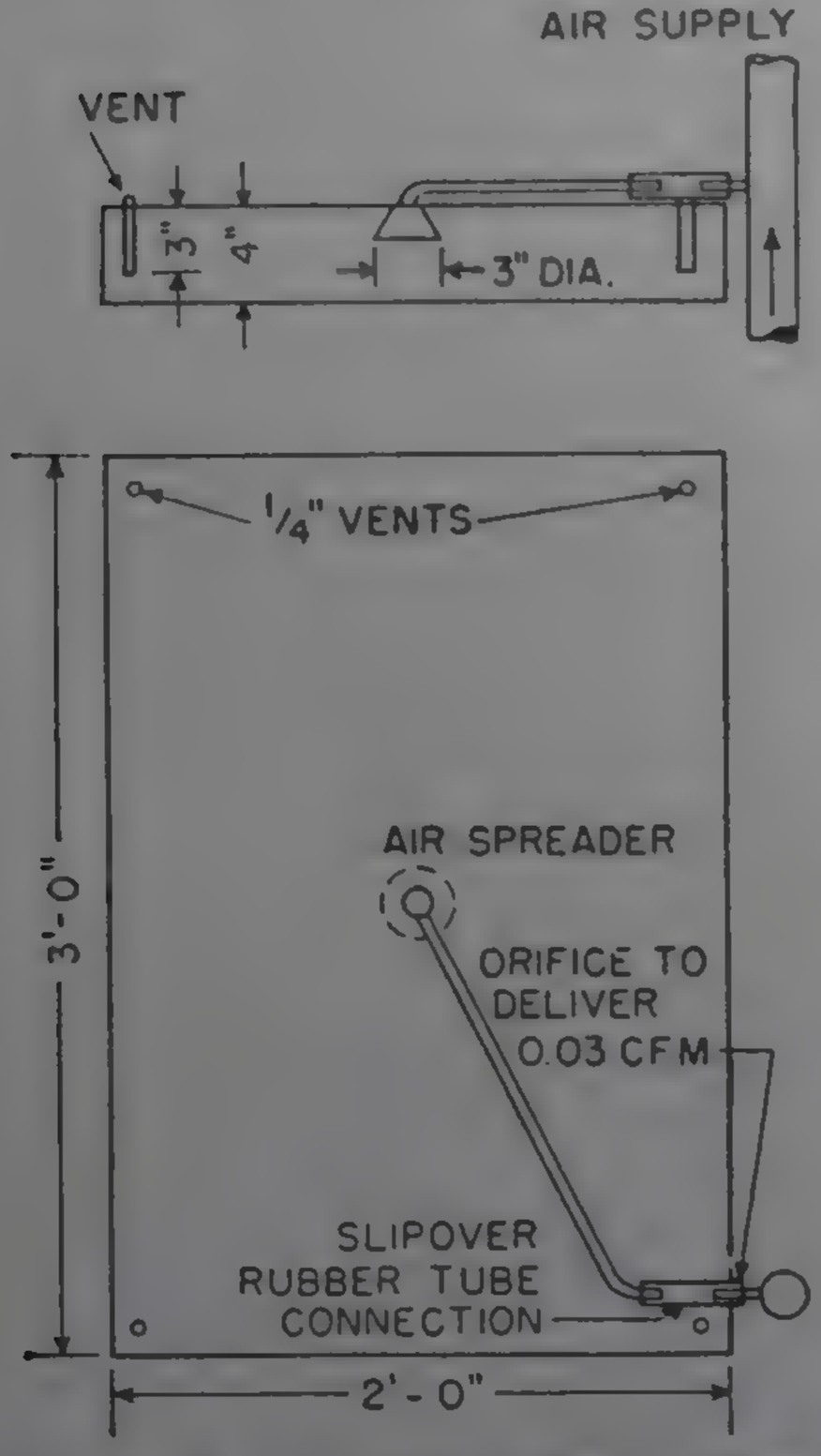


FIG. 12.—Inoculum pan. [*Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem.* **24** (No. 1): 1 (1947).]

Cooling was accomplished by blowing air through the mass, with the mixer in operation, until its temperature reached about 35°C.

The moist bran was inoculated by blowing a spore culture onto the mass and continuing agitation of the mixer.

INOCULUM DEVELOPMENT.—The inoculum, or spore culture, was prepared in special galvanized iron pans, measuring 24 by 35 by 4 in. and equipped with covers containing a central air inlet and air outlets at each corner (refer to Fig. 12). These pans were especially designed to provide adequate aeration of the culture, optimum thickness of the bran layer, and some control of the humidity.

Each pan received 4.5 lb. of the standard corn-bran-acid mixture (refer to p. 70), which was uniformly distributed to form a layer about 0.5 in. deep. The pans and their contents were sterilized with steam and cooled. The moist bran was then inoculated with mold spores and incubated at 32°C. in a constant temperature cabinet. Air at a rate of 1,200 to 1,800 ml. per min. and at a temperature of 30 to 32°C. was admitted to each tray through the central inlet. When sporulation was at an optimum, usually at the end of 4 or 5 days, the cover of the pan was removed and the culture was allowed to air dry in the cabinet during a period of 24 hr.

INOCULATION METHODS.—Underkofler and his coworkers[1] studied the uses of mycelium and of spore inoculums. Mycelium inoculums could be used, but were inconvenient to handle and tended to become too contaminated for further use after two or more transfers. Spore inoculums

[1] *Ibid.*

proved to be very satisfactory and as little as 0.04 per cent of dry spore culture could be used successfully. However, an inoculum consisting of 0.1 per cent of dry spores was used for subsequent pilot-plant studies in order to provide a reasonable factor of safety against variations in spore viability.

INCUBATION METHODS.—Underkofler, Severson, Goering, and Christensen[1] studied extensively a number of different incubation methods in their search for a pilot-plant procedure that would be rapid and practical. They found that the tray method was simple to employ, but that there

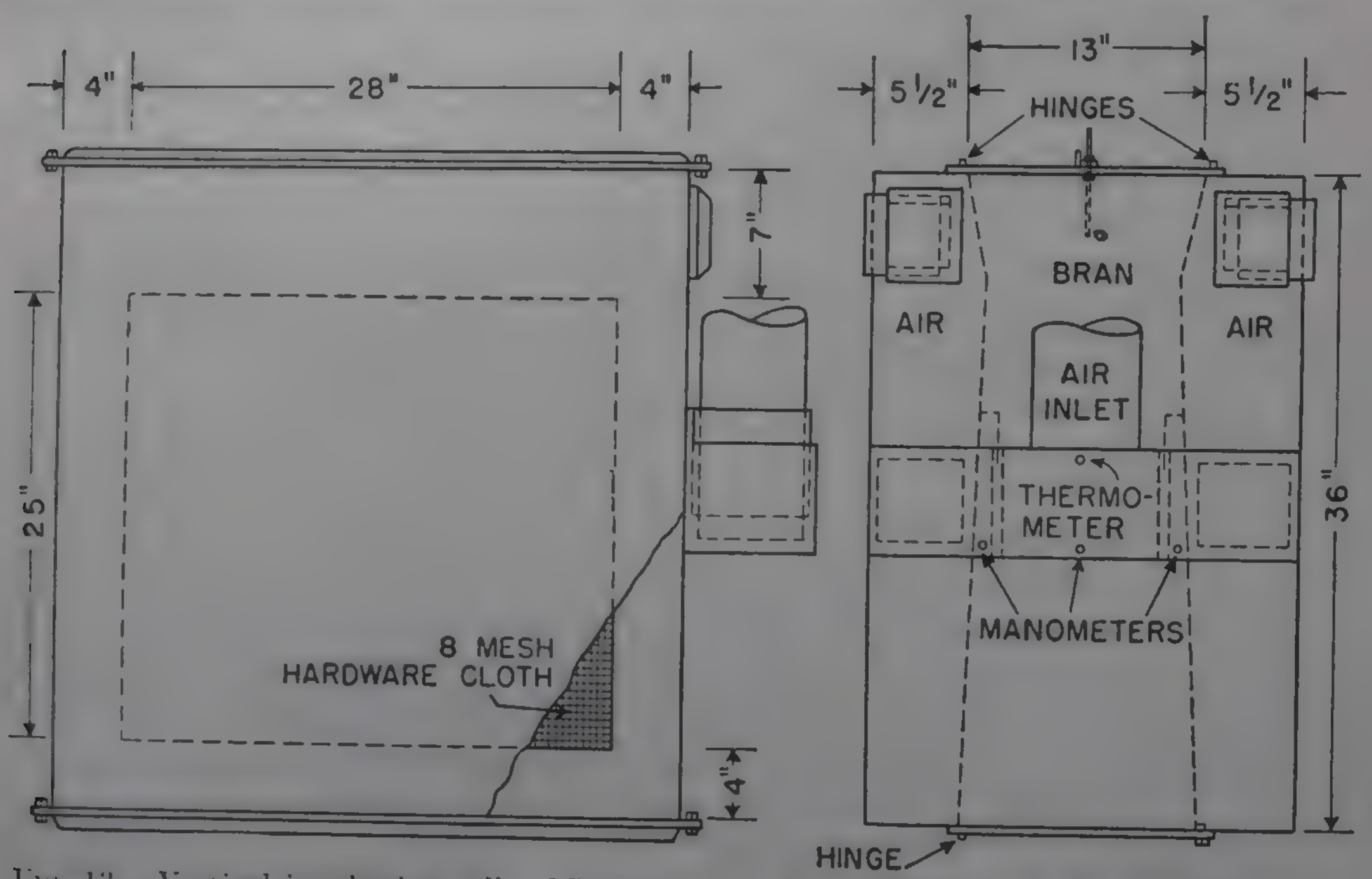


FIG. 13.—Vertical incubation cell. [*Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem.*, **24** (No. 1): 1 (1947).]

were problems associated with the use of the large number of trays required. In this method, it was not efficacious to pile the inoculated bran in layers over 2 in. deep in the pans, for the mold did not grow throughout the mass in the deeper layers. Since the temperature of the bran generally commenced to rise in about 5 to 6 hr. after its inoculation, it was necessary at this point to commence aeration of the mass with air nearly saturated with moisture.

Studies were carried out using specially designed incubation cells and forced aeration. Vertical and inclined cells (refer to Figs. 13 and 14), equipped with hardware cloth faces, proved to be satisfactory. It was found that the temperature of incubation and the moisture content of the bran were dependent variables. The mold grew most rapidly in the bran

[1] *Ibid.*

when the moisture content was adjusted suitably, between 32 and 38° C. The optimum moisture content resulted when 8 parts of 0.1 N hydrochloric acid were used with 10 parts of bran (by weight). Under these conditions a bran with an initial moisture content of 12 per cent contained

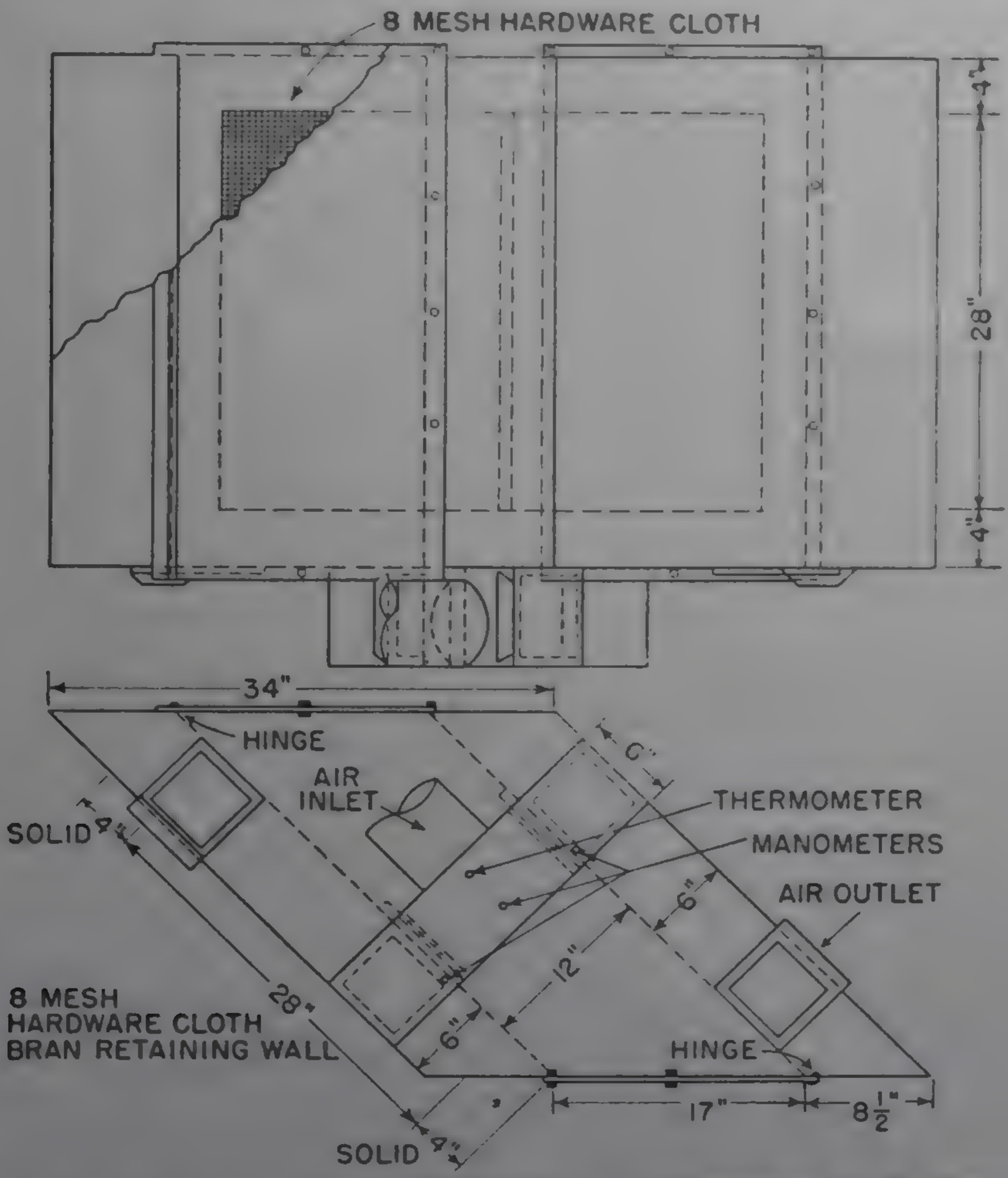


FIG. 14.—Inclined incubation cell. [*Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem.*, **24** (No. 1): 1 (1947).]

about 51 per cent of moisture at the time it was placed in the incubator. Two-stage incubation appeared to be advantageous.

DRYING.—Mold bran from the incubation cells, containing 25 to 30 per cent of moisture, may be dried in layers up to 4 ft. in depth in bins constructed with false bottoms through which dry air is blown. Underkofler and his associates[1] have pointed out that the air temperatures should not be in excess of about 45°C. (113°F.) if serious losses in amylolytic activity were to be avoided.

[1] *Ibid.*

Semicommercial Production Methods.—An installation for producing mold bran on a semicommercial scale was constructed at the alcohol plant of the Farm Crops Processing Corporation (Omaha, Neb.). A cooker of similar design but somewhat larger than the one described on p. 73 was used. Primary incubation was carried out in inclined cells, secondary incubation in closed bins containing bottoms of perforated metal. Difficulties were encountered in connection with the usage of the primary incubation cells, for air control was found to be critical and temperature

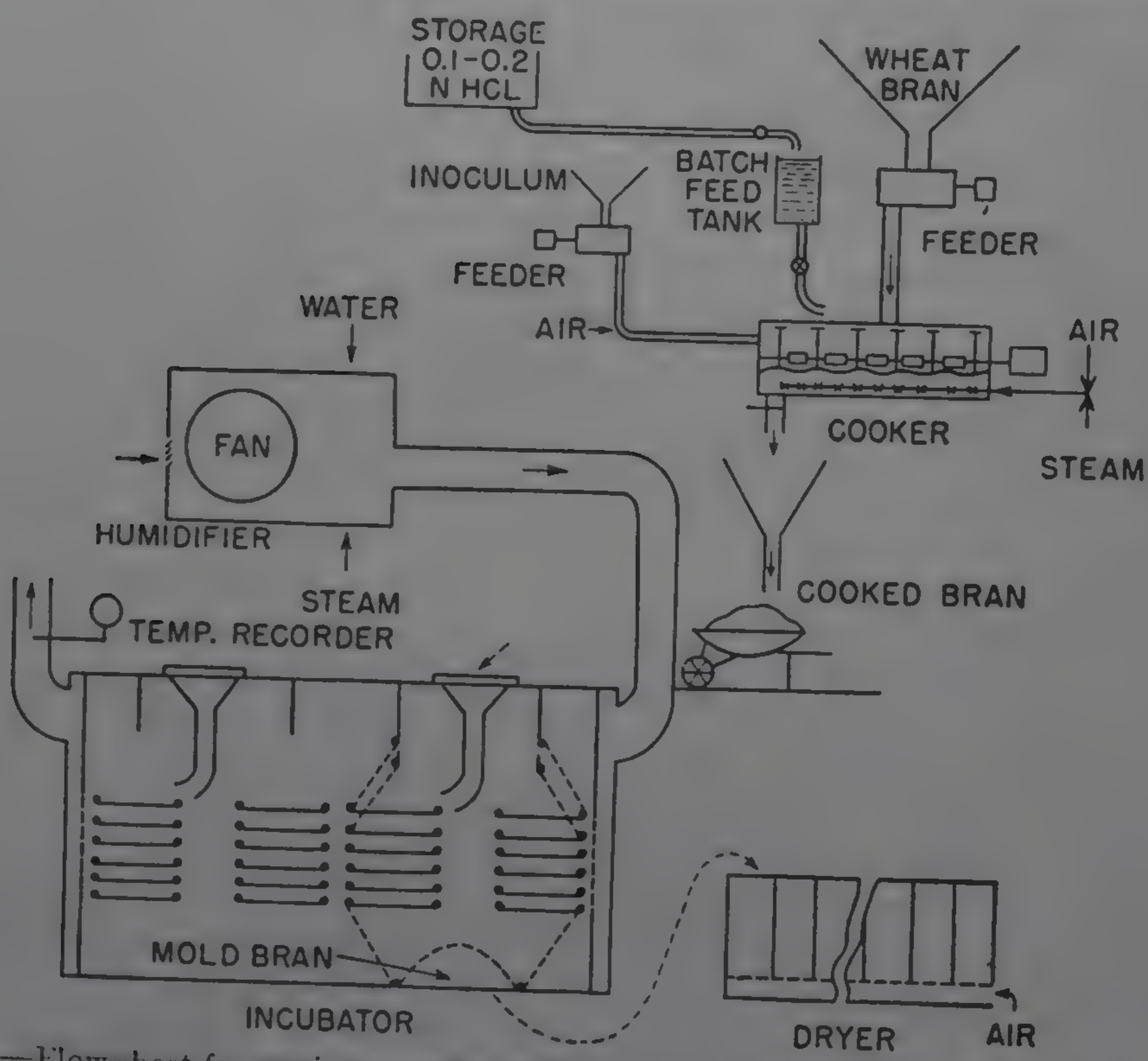


FIG. 15.—Flow sheet for semicommercial mold-bran production. [*Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem.*, **24** (No. 1): 1 (1947).]

control very difficult to achieve. In addition, shrinkage of the bran created air leakages at the top of the cells. The mold bran produced in this unit with careful control was fair in quality.[1]

While the first unit was in production, a successful procedure for handling trays was devised, which led to the construction of a new incubator room (refer to Fig. 15). Trays, measuring about 5 by 14 ft., were hinged lengthwise in such a manner that the front could be dropped down for discharging mold bran and raised for filling purposes. The trays were suspended in a special incubator room constructed of tile and supplied

[1] *Ibid.*

with air ducts for circulating humidified air at the proper temperature. The secondary incubator, previously used in the first unit, was employed to dry the mold bran. The difficulties concerned with control were absent in this unit. Likewise, mold growth was rapid and the quality was uniformly good.[1]

Commercial Production Method.[2]—Wheat bran and dilute hydrochloric acid (0.1 to 0.2 N) are mixed and heated to 100°C. with direct steam, at which temperature the mixture is held for 30 min. with continuous agitation. The temperature of the cooked bran is reduced to 32.2°C. (90°F.) through the use of a stream of air. Spores of *A. oryzae* are thoroughly mixed with the bran, which contains approximately 50 per cent moisture and has a pH of about 3.5. The inoculated bran is distributed rather uniformly on swinging trays (arranged to facilitate easy filling and removal of the mold bran) in special large-sized incubators. Humidified air at the proper temperature is caused to flow over and under the trays until the maximum amount of mold growth has taken place, which generally requires about 36 hr. The mold bran thus prepared is dropped from the trays and air-dried to a moisture content of approximately 12 per cent. One commercial product is sold under the trade name of "Eaglezyme."

Preparation and Saccharification of Mashes.—Mashes may be prepared with or without the use of acids, enzymes, and steam under pressure. A description follows of some of the methods now used in the laboratory or on a commercial basis.

Method of Hao, Fulmer, and Underkofler.[3]—Sixty grams of corn meal and 300 ml. of 0.04 N hydrochloric acid are placed in a 500-ml. Erlenmeyer flask and thoroughly mixed. The starch of the corn is gelatinized by heating the mixture (with occasional agitation) over a low flame or on a hot plate.

The mash is then placed in an autoclave and treated with steam at a pressure of 20 lb. per sq. in. for 30 min. The pH of the cooked and sterilized mash is adjusted to 4.5 to 5.0 with sodium hydroxide, sodium carbonate, or calcium carbonate. The mash, at a temperature of 30°C., is placed in a mixer and mold bran is added as a slurry in water. After agitation for 1 min., the mixture is returned to the 500-ml. Erlenmeyer flask and incubated for 1 hr. at 30°C. for saccharification of the soluble starches. A higher temperature (55°C.) and varying times (1 to 3 hr.) were also used in some experiments for conversion. (Hao and his associates inoculated a mash of the size mentioned above with 20 ml. of a

[1] *Ibid.*
[2] BOYER and UNDERKOFLER, *loc. cit.*
[3] HAO, FULMER, and UNDERKOFLER, *loc. cit.*

24-hr. culture of a special strain of *S. cerevisiae* in 10 per cent malt extract broth.)

Method of Roberts, Laufer, Stewart, and Saletan.[1]—Two different types of cookers were used by Roberts and his associates. These are described below under the headings of pressure mashing and atmospheric mashing.

PRESSURE MASHING.—To 400 ml. of water which has been heated to 70° C. are added 97 g. of wheat and 0.5 g. of dry mold bran. The mash is held, with constant stirring, at 50° C. for 15 min. and then the temperature is raised during a 15-min. period to 60° C. at which temperature it is held for 30 min. The temperature of the mash is then raised to 93° C. during a 15-min. period and held at 93° C. for 30 min. The mash is heated finally with steam for 1 hr. at a pressure of 20 lb. per sq. in. and cooled to the temperature of conversion.

ATMOSPHERIC MASHING.—This procedure is identical with that of pressure mashing up to the point where the temperature reaches 66° C. The mash is held at 66° C. for 55 min. and cooled rapidly (within 5 min.) to the saccharification temperature.

Saccharification is carried out at either 30 or 52.5° C. Mold bran (Takadiastase) is prepared as a slurry in a concentration of 2.5 to 4 per cent. The combined weight of grain and saccharifying agent is always maintained at 100 g.—and held for 15 min. at the saccharification temperature. The slurry is then added to the mash and saccharification effected by holding it for 30 min. at the specified saccharification temperature.

Commercial-scale Method.—Underkofler, Severson, and Goering[2] have reported on the use of mold bran on a commercial basis at the alcohol plant of the Farm Crops Processing Corporation, Omaha, Nebraska. The method used for preparing and saccharifying the mashes was as follows:

The grain is ground and prepared as a slurry, using 1.5 gal. of liquid per bushel of grain at 15° C. (60° F.). The slurry is passed through pressure cookers where the temperature is raised to approximately 66° C. (150° F.) by the direct injection of steam. It is then cooked for 5 min. at 106° C. (223° F.) at a gauge pressure of 120 lb. The temperature of the cooked mash is reduced to 77° C. (170° F.) by flashing the mash through two pressure-reducing chambers, one at a gauge pressure of 16 lb. and the other under vacuum.

The mold bran is prepared with cold water as a slurry containing the required concentration (usually 4 per cent) for saccharification. Next the slurry is mixed into a stream of hot mash, reducing the temperature of the mixture to 61° C. (142° F.). The mixture is held for approximately 2 min.

[1] ROBERTS, LAUFER, STEWART, and SALETAN, *loc. cit.*

[2] UNDERKOFLER, SEVERSON, and GOERING, *loc. cit.*

at this temperature and is then pumped through coolers to the fermenters where it enters at a temperature of about 28°C. (82°F.). It requires about 3 hr. to fill a fermenter of 130,000-gal. capacity. About 30 min. after filling has begun, yeast is pumped into the fermenters. The temperature is maintained at a maximum of 35°C. (95°F.) in the fermenter by the continuous circulation of the mash through external coolers. Thus saccharification takes place largely in the fermenter where the initial pH is about 5.2 and the concentration 22.4 g. per 100 ml., or 30 gal. of liquid per bushel of grain.

Effect of Conditions of Saccharification on Alcohol Yields.—The method used in preparing the grain mashes, the pH and concentration of the the mashes during saccharification, the nature and concentration of the mold bran used, the temperature and time of saccharification, and other factors, exert an important influence on the subsequent fermentation and yields of alcohol obtained. Several of these factors have already been discussed in the preceding paragraphs. Some additional information will be presented now.

Roberts and his coworkers[1] found that maximum alcohol yields were obtained with mold-bran saccharification when the mashes were cooked at atmospheric pressure.

Naturally there is no one pH value that is optimum under all conditions of saccharification. However, the use of a pH somewhere in the range of 4.5 to 5.0 is usually suitable for saccharification, as it favors particularly the activities of the amylolytic enzymes, α-amylase and β-amylase.

The concentration of mash at the time of saccharification depends upon the method used. The weight of grain usually represents about 16 to 20 per cent of the weight of the total mash.

Hao and his associates[2] concluded that saccharification of corn mashes for 1 hr. at 30°C. with mold-bran preparations was as satisfactory as employing 1 to 3 hr. at 55°C. (Refer to Table 11 on page 70.)

The usual concentration of mold bran employed for saccharification ranges from 2.5 to 4.0 per cent, depending upon the amylase activity of the product. A 4.0 per cent concentration based on the weight of grain used appears to produce most satisfactory results in terms of yields of ethyl alcohol.

Roberts and his collaborators[1] showed that mold-bran saccharification at 30°C. was equal to or slightly better than at 52.5°C. both in the case of mashes cooked at atmospheric pressure (66°C.) and in the case of those cooked with pressure (125°C.). They also demonstrated that the pre-

[1] Roberts, Laufer, Stewart, and Saletan, *loc. cit.*

[2] Hao, Fulmer, and Underkofler, *loc. cit.*

malting of mashes with barley malt increased their liquefaction but did not result in improving the yields of alcohol over those saccharified with only 3 to 4 per cent mold bran.

Comparison of Mold Bran with Malt as a Saccharifying Agent.—The most suitable means for evaluating amylolytic agents is based upon comparisons of the yields of ethyl alcohol recovered from the saccharified grains. A number of such comparisons have been made. A few typical results and conclusions are included below.

Table 12 shows the results of plant-scale tests of mold bran at the Farm Crops Processing Corporation, Omaha, Neb. In the tests cited, mold bran was used as a replacement of supplemental malt in preparing yeast culture mashes, with the result that the yields of alcohol were increased. This use of mold bran has resulted also in a reduction of the time required for lactic souring (*i.e.*, acid production by lactic acid bacteria) and higher yeast counts.[1]

TABLE 12.—RESULTS OF PLANT-SCALE TESTS OF MOLD BRAN[1]

No. of fermenters	Per cent of saccharifying agent in fermenter mashes	Per cent of saccharifying agent in yeast culture mashes	Av. alcohol yield/standard bu.	
			Proof, gal.	190-proof, gal.
299	10 malt	22 malt	4.77	2.51
847	9–10 malt	8.6 malt + 4.3 mold bran	5.17	2.72
6	4 mold bran	8.6 malt + 4.3 mold bran	5.24	2.76
12	9–10 malt	8.6 malt + 4.3 mold bran	5.15	2.71
7	3.9–6.2 malt + 2.2–0.9 mold bran	8.6 malt + 4.3 mold bran	5.26	2.77
12	9–10 malt	8.6 malt + 4.3 mold bran	5.23	2.75

[1] UNDERKOFLER, L. A., G. M. SEVERSON, and K. J. GOERING, *Ind. Eng. Chem.*, **38**: 980 (1946).

Table 13 shows results obtained by Roberts and his associates using different quantities of malt and mold bran as the saccharifying agents for corn, two kinds of wheat, and two kinds of granular wheat flour. The mashes were prepared as indicated in the previous section which was concerned with the preparation and saccharification of mashes.

A Comparison of Mold Preparations as Conversion Agents.—Hao and Jump[2] have compared some crude, intermediate, and purified mold preparations for their conversion efficiencies and yields of ethanol from corn mash. The preparations used by them are listed in Table 14. The

[1] UNDERKOFLER, SEVERSON, and GOERING, *loc. cit.*

[2] HAO, L. C., and J. A. JUMP, *Ind. Eng. Chem.* **37**: 521 (1945).

TABLE 13.—YIELD OF ALCOHOL IN PROOF GALLONS PER 100 LB. OF GRAIN ON DRY BASIS[1,2]

Saccharifying agent	Conversion temp., °C.	Corn	Winter wheat		Spring wheat		Winter granular wheat flour		Spring granular wheat flour	
		Pressure	Pressure	Atmospheric	Pressure	Atmospheric	Pressure	Atmospheric	Pressure	Atmospheric
8 % malt	57.5	10.37	10.05	9.37	9.68	9 53	11.69	11.19	11.58	10.90
1 % malt[3] + 2 % mold bran	52.5	10.36	8.92	10 45	9.04	10.09	11.89	12.25	11.67	12.17
	30.0	10.34	9.57	10.35	9.80	10.10	11.35	12.39	11.87	12.42
2.5 % mold bran	52.5	10.12	7.76	9.52	8.85	10.22	11.02	12.15	11.21	12.13
	30.0	10.43	9.58	9.82	9.38	10.18	11.19	12.22	11.66	12.43
3 % mold bran	52.5	10.50	7.96	10.10	9.24	10.35	.....	12.42	.....	12.39
	30.0	.....	.....	10.34	....	.....	.....	.....	.....	12.24
4 % mold bran	52.5	.....	.....	10.17	....	9.97	.....	12.40	.....	12.70
3 % wet mold bran[4]	52.5	9.93	.....	10.25	....	9.73	.....	12.47	.....	12.35
	30.0	10.24	.....	9.83	....	9.20	.....	11.96	.....	11.68

[1] ROBERTS, M., S. LAUFER, E. D. STEWART, and L. T. SALETAN, *Ind. Eng. Chem.*, **36**: 811 (1944).

[2] Yields are corrected for alcohol produced from malt. Alcohol from mold bran is negligible. All yields are averages of two or more results.

[3] For premalting.

[4] Sample contained 50 per cent moisture; therefore, 6 g. were used.

TABLE 14.—SOURCES OF MICROBIAL AMYLASE PREPARATIONS[1]

Name of Sample	Producer
Bacterial amylase I	[2]
Bacterial amylase II	Wallerstein Co., Inc.
Bacterial amylase III	[2]
Mold bran I	Frederick Stearns & Co.
Mold bran II	Wallerstein Co., Inc.
RHozyme, Maltase No. 20, RHozyme Intermediate, Maltase Intermediate	Rohm & Haas Co., Inc.
Converzyme No. 503, Converzyme No. 505	Joseph E. Seagram & Sons, Inc. (lab. preparation)
Converzyme No. 511	[2]
Polidase-C, Polidase-S	Schwarz Lab., Inc.
Diatane type 1211, Diatane B-10	Jeffrey Lab.
Taka-Diastase	Parke, Davis & Co.
Alase	Takamine Lab.
Crystazyme	Frederick Stearns & Co.

[1] HAO, L. C., and J. A. JUMP, *Ind. Eng. Chem.*, **37**, 521 (1945).

[2] Name of producer not revealed because of limited supply of product or other technicalities.

mashes contained, in addition to the conversion agents, 91 parts of corn and 1 part of liquefying material (malt), unless otherwise indicated.

As a result of their studies, Hao and Jump made the following conclusions: All the crude mold-amylase preparations studied (Converzymes 503, 505, and 511; mold brans I and II; Diatane T-1211; and Polidase-C) were efficient agents for converting corn mash, the conversion efficiency being higher than that of malt but the yields of alcohol being about the same. The intermediate mold-amylase preparations were excellent agents for conversion and produced thinner mashes than malt and crude amylase preparations. The conversion efficiencies and yields of alcohol (based on total grain) were higher with 4 parts of RHozyme Intermediate or 6 parts of Maltase Intermediate than with 8 parts of malt. The purified mold-amylase preparations (RHozyme, Maltase No. 20, Taka-Diastase, Polidase-S, Alase, and Crystazyme) were also excellent conversion agents, 0.25 to 1.5 parts of the agents producing satisfactory conversion.

The value of mixing malt with mold-amylase preparations may be ascertained by reference to Table 15. The value of hydrochloric acid as a

TABLE 15 COMPARISON OF ALCOHOL YIELDS FROM CORN-MASH-CONVERTED PREPARATIONS AND 1 PER CENT MALT[1]

Run No	Conversion material	Mash composition, wt. ratio[2]		Conversion efficiency, per cent	Yield, proof gal./bu., based on	
		Conversion material	Liquefying material (malt)		Corn	Total grain
631	Malt	8	1	88.6	5.86	5.73
702	Diatane T-1211 control	5	1	90.0	5.93	5.74
731	Diatane T-1211 + Malt	5 + 1	1	94.0	6.20	5.94
741	Diatane B-10 + Malt	5 + 1	1	94.6	6.24	5.98
733	Diatane B-10	5	3*	96.5	6.37	6.15

[1] Hao, L. C., and J. A. Jump, *Ind. Eng. Chem.*, **37**, 521 (1945).
[2] [illegible] on dry basis.
* Concentrated hydrochloric acid.

liquefying agent is also indicated. (The disadvantage of using acid for this purpose lies in the fact that the mash must be neutralized before the conversion agent is added.)

Hao and Jump[1] reported that the addition of the mold-amylase

[1] Hao, L. C., and J. A. Jump, *Ind. Eng. Chem.*, **37**: 521 (1945).

preparation at intervals increased the efficiency of conversion and the yield of ethyl alcohol by 1 to 2 per cent. About three-fifths of the preparation was added at the conversion time and the remaining portion after the fermentation had proceeded for 20 hr.

SUBMERGED-CULTURE PRODUCTION OF MOLD AMYLASES

Considerable interest has been shown recently in the production of fungal amylases by submerged-culture methods. These enzyme products are manufactured on the premises for use as a malt substitute or supplement in the conversion of grains for such purposes as the production of industrial alcohol. The amylases are generally used without concentration or purification and may be produced on a number of media.

Production.—The production of fungal amylases by submerged-culture methods has been studied by Balankura, Stewart, Scalf, and Smith;[1] Van Lanen and Le Mense;[2] Le Mense, Corman, Van Lanen, and Langlykke;[3] Adams, Balankura, Andreasen, and Stark;[4] and others.

Production involves growing a high amylase-producing strain of mold in a suitable medium under controlled conditions of temperature, pH, and aeration.

Organisms Used.—Van Lanen and Le Mense,[2] and Le Mense and associates[3] studied the production of amylases in a thin stillage medium by 367 cultures of molds, which included species of the genera *Penicillium*, *Aspergillus*, *Rhizopus*, *Mucor*, and *Monilia*. Only 10 per cent of the 80 cultures of the genus *Penicillium* examined demonstrated dextrinizing activity and this was of low order. Cultures of the genera *Rhizopus*, *Mucor*, and *Monilia* produced either very little or no dextrinizing enzyme. Greatest activity was observed in cultures of the genus *Aspergillus*. Of 278 cultures studied, 34 produced the dextrinizing enzyme. Highest concentrations of this enzyme were produced by strains of *A. niger*, *A. oryzae*, *A. alliaceus*, *A. foetidus*, and *A. wentii*. Some strains of *A. niger* produced both dextrinizing and saccharifying enzymes. Among these was *A. niger* NRRL 367 which exhibited the greatest potency of any of the cultures examined by the Northern Regional Research Laboratory group.

Media.—Various media may be used for the production of fungal amylases by submerged-culture methods. Le Mense and his associates[5]

[1] Balankura, B., F. D. Stewart, R. E. Scalf, and L. A. Smith, *Jour. Bact.*, **51** (No. 5): 594 (1946).

[2] Van Lanen, J. M., and E. H. Le Mense, *Jour. Bact.*, **51** (No. 5): 595 (1946).

[3] Le Mense, E. H., J. Corman, J. M. Van Lanen, and A. F. Langlykke, *Jour. Bact.*, **54** (No. 2): 149 (1947).

[4] Adams, S. L., B. Balankura, A. A. Andreasen, and W. H. Stark, *Ind. Eng. Chem.*, **39**: 1615 (1947).

[5] Le Mense, Corman, Van Lanen, and Langlykke, *loc. cit.*

used a medium that contained thin stillage (a product of the alcoholic fermentation of corn and sorghums), 2 per cent of corn meal, and 0.5 per cent of calcium carbonate. The culture liquors obtained after growth of the mold for 3 to 5 days with continuous aeration were satisfactory substitutes for malt in the conversion of corn mashes.

The relative values of other media for the production of the dextrinizing enzyme by *A. niger* NRRL 337 are indicated in Table 16.

TABLE 16.—THE PRODUCTION OF DEXTRINIZING ENZYME BY *Aspergillus niger* NRRL 337 CULTIVATED IN VARIOUS MEDIA[1,2]

Protein source	Carbohydrate source	Concentration of dextrinizing enzyme, units/ml.
Corn steep liquor, 3%	None	2.2
Corn steep liquor, 3%	Glucose, 2%	8.2
Corn steep liquor, 3%	Molasses, 2%	4.6
Corn steep liquor, 3%	Corn meal, 2%	10.2
Dried tankage, 2%	None	2.1
Dried tankage, 2%	Glucose, 2%	9.3
Dried tankage, 2%	Molasses, 2%	11.5
Dried tankage, 2%	Corn meal, 2%	8.7
Soybean meal, 2%	None	7.9
Soybean meal, 2%	Glucose, 2%	7.4
Soybean meal, 2%	Molasses, 2%	8.5
Soybean meal, 2%	Corn meal, 2%	11.2
Thin stillage	None	1.7
Thin stillage	Glucose, 2%	11.5
Thin stillage	Molasses, 2%	7.9
Thin stillage	Corn meal, 2%	16.5
Thin stillage	Xylose, 2%	5.3
Thin stillage	Lactose, 2%	6.7
Thin stillage	Sucrose, 2%	11.0
Thin stillage	Maltose, 2%	14.5

[1] DE MESSE, E. H., J. CORMAN, J. M. VAN LANEN, and A. F. LANGLYKKE. *Jour. Bact.*, **54** (No. 2): 140. 1947.

[2] Inoculated flasks containing 50 ml. of the various media were shaken for 5 days. Composition of medium: protein and carbohydrate as shown plus 0.5 per cent of calcium carbonate.

Adams, Balankura, Andreasen, and Stark[1] employed a medium that contained 5 g. per 100 ml. of distillers' dried solubles (stillage from the distillation column, screened to separate out most of the suspended solids, evaporated to about 30 per cent solids and then dried on drum driers) and 1 g. of corn meal per 100 ml. Distillers' dried solubles proved to be

[1] ADAMS, BALANKURA, ANDREASEN, and STARK, *loc. cit.*

better than stillage for mold amylase production. The pH of this medium was adjusted to 5.0 with calcium carbonate. Adams and his collaborators reported that the mold amylase produced in this medium could be used instead of barley malt, and that the yield of ethyl alcohol was actually 0.2 to 0.3 proof gal. per bu. of grain greater than when malt was employed.

Conditions of Production.—Le Mense and his associates[1] dispensed 50-ml. amounts of medium in 200-ml. flasks and 200-ml. amounts in 1-liter flasks. These were sterilized with steam for 30 min. at 20 lb. pressure. After cooling and inoculation, they were incubated at 30°C. and shaken continuously in a Kahn-type shaker (ninety 3-in. strokes per min.). 4-liter amounts of medium were used for aeration studies in 8-liter Pyrex cylinders, which were equipped with air spargers built of perforated aluminum tubing and had aluminum plate lids. These containers plus their contents were sterilized for 1 hr. at 25-lb. pressure.

Adams and his collaborators[2] sterilized 1-liter portions of their medium in 2-liter, cone-shaped flasks. In building up the inoculum, the medium was inoculated the first time with 2.0 per cent by volume of a sporulated culture (grown by surface method) of *A. niger* NRRL 337. The flask was incubated at 30°C. in a water bath and the contents were aerated for 24 hr. with 0.2 cu. ft. per min. per liter of air, using an Aloxite sparger. Thereafter the mold culture was transferred serially through a number of 24-hr. submerged stages (usually two), using 0.5 per cent by volume of inoculum for each succeeding mash. The final stage was aerated for 48 hr.

Methods Used for Evaluating Submerged Fungal Amylase Preparations.—These are concerned with a determination of the dextrinizing and saccharifying activities of the enzymes and of the alcohol produced from grain mashes converted by them and subsequently fermented by yeasts. Adams, Balankura, Andreasen, and Stark[2] made three determinations: α-amylase activity by the 30°C. method of Sandstedt, Kneen, and Blish;[3] the saccharifying power by an unpublished method of Hao; and the alcohol yields by the method of Stark and his associates.[4] Le Mense and his associates[5] determined the dextrinizing activity by the method of Sandstedt, Kneen, and Blish[3] (as modified by Olson, Evans and Dickson[6]); and the maltase activity by calculating the increase in reduc-

[1] Le Mense, Corman, Van Lanen, and Langlykke, *loc. cit.*

[2] Adams, Balankura, Andreasen, and Stark, *loc. cit.*

[3] Sandstedt, R. M., E. Kneen, and M. J. Blish, *Cereal Chem.*, **16**: 712 (1939).

[4] Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov, *Ind. Eng. Chem., Anal. Ed.*, **15**: 443 (1943).

[5] Le Mense, Corman, Van Lanen, and Langlykke, *loc. cit.*

[6] Olson, W. J., R. Evans, and A. D. Dickson, *Cereal Chem.*, **21**: 533 (1944).

ing power, found by Somogyi's method[1] after incubating a 10-ml. portion of culture filtrate with 20 ml. of a 1.05 per cent maltose solution for 2 hr. at 30°C. (The mixture was kept at a pH of 4.6 by the addition of an "acetate buffer to the maltose solution.")

Mashing and Conversion Methods.—The method of Le Mense *et al*[2] was as follows: Into each of a series of 500-ml. Erlenmeyer flasks were placed 49.5 g. of ground corn, 0.5 g. of ground barley malt, and 170 ml. of tap water warmed to 70°C. The flasks were incubated in a water bath at 70°C. and the mashes were stirred intermittently for 30 min. for premalting. The mashes were then cooked for 30 min. at a steam pressure of 25 lb. per sq. in. and cooled to 75°C. The mold enzyme liquor was added to the mash with enough water to lower the temperature to 55 to 56°C., at which temperature conversion was carried out for 30 min. with frequent agitation of the mashes. The same method was used for malt controls, except that 45 g. of corn and 5 g. of malt were used, 0.5 g. of the latter being for premalting and 4.5 g. for conversion.

In the procedure of Adams and his associates,[3] the grain to be converted (corn) was cooked for 1 hr. at atmospheric pressure, autoclaved for another hour at 22 lb. pressure, and cooled to the temperature of conversion. Sufficient submerged mold culture was added to the mash to represent 10 per cent of its final volume. The mash was agitated for 5 min. and cooled to the temperature of setting. Ten per cent by volume of stillage was then added, and the pH was adjusted to 4.8 and the total volume to 38 gal. per bu. of grain. The mash was converted at a temperature of 145°F.

Fermentation.—The corn mashes converted by the method of Le Mense and coworkers were inoculated in the proportion of 2 per cent by volume with a 24-hr. culture of distillers' yeast (NRRL Y567), after being cooled to 30°C., the temperature of fermentation. The final volume in the fermentation flask was about 250 ml. in each case. The fermentations were allowed to continue for 72 hr. The fermented mashes (beers) were made up to a volume of 300 ml., of which a 200-ml. aliquot was distilled. In each instance 100 ml. of distillate were collected. The alcohol content was ascertained by determination of the refractive index.

Yields.—Yields of alcohol obtained by the procedure of Adams and his associates varied from 6.16 to 6.27 proof gal. per bu., as compared with 5.95 proof gal. per bu. for the malt-converted control.

[1] SOMOGYI, M., *Jour. Biol. Chem.*, **160**: 61 (1945).
[2] LE MENSE, CORMAN, VAN LANEN, and LANGLYKKE, *loc. cit.*
[3] ADAMS, BALANKURA, ANDREASEN, and STARK, *loc. cit.*

Some Factors Affecting Amylase Production.—Erb, Wisthoff, and Jacobs[1] investigated some of the factors that influence amylase production by *A. niger* NRRL 337. The basic medium used by them was a 10 per cent corn mash, acidified to a pH of 4.0 with sulphuric acid and cooked for 1 hr. at a steam pressure of 30 lb. The concentration of the medium was adjusted as desired, and calcium carbonate was added to bring the pH to 5.3. Urea, in the proportion of 0.25 per cent, was added to each flask. The medium was distributed in 100-ml. amounts in 1-liter Erlenmeyer flasks, sterilized, cooled, and inoculated with the spores of *A. niger* NRRL 337 and shaken continuously at 30 to 32°C. for the desired period of time.

It was found that the addition of small quantities of sodium chloride or potassium chloride (0.001 g. per 100 ml.) stimulated amylase production.

Enzyme production was correlated with the concentration of the grain in the medium. When an incubation period of 96 hr. was used, the enzyme production of a medium with an initial concentration of 10° Brix was decided superior to that of media of 8 and 6° Brix concentrations, respectively. However, based on an incubation period of 22 hr., the medium of 6° Brix concentration possessed greater enzyme activity than the media of greater concentration.

Sodium pentachlorphenate (Dowicide G), in a concentration of 0.006 to 0.012 g. per 100 ml. of medium, and ammonium bifluoride, in a concentration of 0.015 to 0.02 g. per 100 ml. of medium, may be used to control bacterial contamination during amylase production by *A. niger* NRRL 337, according to Erb, Wisthoff, and Jacobs. Sodium pentachlorphenate tends to inhibit or prevent sporulation of the mold. However, low amylase production and sporulation in shake-flask cultures are frequently correlated.

Action of Mold Enzymes in the Saccharification of Starch.—Corman and Langlykke[2] studied the action of the amylolytic enzymes found in mold culture filtrates on the saccharification of starch. The action appeared to be due principally to an α-amylase and to a glucogenic enzyme system. Little or no β-amylase was observed in the mold culture filtrates. The action of the fungal α-amylase was related to that of malt α-amylase. The α-amylase liquefied starch and formed dextrins and maltose. The glucogenic enzyme system acted upon maltose, dextrins, and starch and formed glucose. This system differed markedly from β-amylase which produces maltose. The efficiency of saccharification

[1] Erb, N. M., R. T. Wisthoff, and W. L. Jacobs, *Jour. Bact.*, **55** (No. 6): 813 (1948).

[2] Corman, J., and A. F. Langlykke, *Cereal Chem.*, 25 (No. 3): 190 (1948).

appeared to be more closely correlated with the activity of the glucogenic enzyme system than with the activity of α-amylase.

USE OF BACTERIAL AMYLASE PREPARATIONS AS LIQUEFYING AGENTS

Bacterial amylase preparations may be used instead of malt for the liquefying of cooked grain mashes in the production of ethyl alcohol, according to Hao and Jump.[1] Table 17 compares alcohol yields from

TABLE 17.—Comparison of Alcohol Yields from Corn Mash Liquefied with Bacterial Amylase Preparations

Mash No.	Liquefying material	Weight ratio: Liquefying material	Weight ratio: Corn	Weight ratio: Water	Conversion efficiency, per cent	Yield, proof gal. bu. based on: Corn	Yield, proof gal. bu. based on: Total grain
101	Malt (control)	1.0	8	21	91.4	6.03	5.66
102	Bacterial amylase I	1.0	8	21	91.5	6.04	5.72
103	"	1.5	8	21	92.9	6.14	5.77
104	"	1.0	5	21	91.0	6.02	5.79
105	"	0.5	5	21	86.8	5.72	5.53
106	Bacterial amylase II	1.0	8	21	93.7	6.18	6.00
107	"	1.5	8	21	93.5	6.17	5.99
108	"	1.0	8	21	92.9	6.14	5.78
109	"	0.5	8	21	91.5	6.04	5.70
110	Bacterial amylase III	3.0[a]	8	21	95.5	6.30	6.10
111	"	3.0	8[b]	21	84.0	5.54	5.44

[a] Weight of malt in this mash [illegible] 37 [illegible].

[b] 8 per cent cornmeal + [illegible] of dry basis.

[c] The pH is adjusted to 5.6 before the addition of bacterial amylase.

[illegible] small pH which the starch liquefying agents were malt, bacterial amylases, and commercial hydrochloric acid, respectively, and in which the saccharifying agent was Concentrase No. 305. It will be noticed that weights of malt used with bacterial amylases were slightly higher than they were the malt control when equal weights of the liquefying agents were used. Actually, the bacterial amylases produced a weight of higher viscosity than the malt. Based on the mean of equal weights of malt, according to Hao and Jump. Bacterial amylase III was a purified fungal amylase.

[1] Hao and Jump, loc. cit.

Table 17 also indicates that concentrated hydrochloric acid was the most efficient agent for liquefying the mash, under the conditions employed, the conversion efficiency being 95.5 per cent when the saccharifying agent was Converzyme No. 505.

THE AMYLO PROCESS

This process is primarily one for converting starch to sugar by the use of selected molds, some of which have the ability to produce small quantities of alcohol from sugar.

The grain to be hydrolyzed is soaked in water for several hours to soften it. It is then mixed with approximately twice its weight of water and heated at a pressure of 4 atmospheres to render the starch soluble. Acidification of the mash with 0.6 to 0.8 parts by weight[1] of hydrochloric or sulphuric acid per 100 parts of grain facilitates the liquefaction of the starch. The sterilized mash is cooled to 40 to 38°C. and inoculated with a pure culture of a *Mucor* or a related mold. *M. rouxii*, *Rhizopus japonicus*, *R. tonkinensis*, and *R. delemar* are some of the molds that have been used in the Amylo process. Sterile air is passed through the seeded mash for 24 hr., while the temperature is maintained at 38°C. The mash is then cooled to at least 33°C. and inoculated with yeast.

By using a modification of the Amylo process, developed by Boulard,[2] starch hydrolysis and fermentation are carried out simultaneously by adding at the same time *Mucor boulard* No. 5 and a yeast. It is claimed that time is saved by the use of the Boulard process.

A MODIFIED AMYLO PROCESS

The use of a modified Amylo process for the saccharification of granular wheat flour mashes in the production of ethyl alcohol has been described by Erb and Hildebrandt.[3] In brief, this process involves the development of a mold inoculum, the preparation of the main mashes, the addition of the mold inoculum and a yeast starter (*Saccharomyces anamensis*) to the mash, and saccharification and fermentation.

Development of the Mold Inoculum.—Saccharification of the starch in the granular wheat flour mashes is brought about by the enzymes produced by the mold. In order to facilitate rapid saccharification of the mashes, it is necessary to build up a volume of inoculum equivalent to 6 to 12 per cent of the volume of the final mash. This is accomplished by

[1] OWEN, W. L., The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine & Liquor Jour.*, June, 1936.

[2] BOULARD, H., Société d'exploitation des procédés, H. Boulard, 1931.

[3] ERB, N. M., and F. M. HILDEBRANDT, *Ind. Eng. Chem.*, **38**: 792 (1946).

carrying the development through three stages, the first of which is initiated in the laboratory and the next two in the plant.

Laboratory Stage.—The mold used is a pure culture of either *Rhizopus delemar* or *R. boulard* and is carried on slants of malt agar. The first step in the development of the inoculum is the inoculation of a special medium in a 100-ml. Erlenmeyer flask with mold spores. The medium contains ingredients in the following proportions: 25 g. of flour, 0.1 g. of ammonium sulphate, and 500 ml. of water. It is dispensed in shallow layers in 100-ml. Erlenmeyer flasks and sterilized at 250°F. (121.1°C.) for 1 hr. The spores produced in one flask are suspended in 3,000 ml. of water and used to inoculate the sterilized medium of the vessel employed in the first plant stage described below.

Plant Stages.—A medium containing the following materials is sterilized for 1 hr. at 250°F. (121.1°C.) in a jacketed steel pressure vessel which has a working capacity of 75 gal.: 20 lb. of ground malt, 200 g. of ammonium sulphate, 5 g. of zinc sulphate, 100 ml. of phosphoric acid, and sufficient water to make 75 gal. The mash is cooled to 90°F. (32.2°C.) and inoculated with the 3,000-ml. suspension of mold spores prepared in the laboratory. The pH of the mash before inoculation is 3.8 to 4.0. The seeded mash is aerated at a pressure of 5 lb. Within a period of 20 hr., there is abundant growth of the mold and the culture is ready for use to inoculate the next larger medium.

In the second plant stage, the medium is prepared and sterilized in a closed steel pressure vessel which has a working capacity of 2,500 gal. and which contains cooling coils. The medium used in this vessel is 500 gal. of granular flour and malt from the mashing system of the plant, 300 gal. of stillage, 0.5 lb. of aluminum powder, 20 g. of zinc sulphate, 6 lb. of ammonium sulphate, 1,000 ml. of sulphuric acid, and sufficient water to make approximately 2,500 gal. The resultant pH of the medium is 3.8 to 4.2. It is sterilized at 250°F. for 1 hr. and subsequently cooled to 95°F. (35°C.). The contents of the vessel from the first stage are blown into this vessel to serve as its inoculum. The seeded medium is aerated at about 5 lb. pressure for 24 hr.

The aluminum powder is used as a constituent of the foregoing medium to counteract a somewhat toxic ingredient of the stillage which appears to inhibit growth of the mold. The action of the interfering substance may be reduced by dilution and it may be removed by treatment with carbon.

Preparation of Main Mashes.—The mashes for conversion by malt and by molds are prepared in a similar manner, except for the conversion agent. The one that is malt-converted contains 190 lb. of flour and 20 lb. of malt per 100 gal. Two antiseptics are added to the mash—0.75 lb.

of ammonium bifluoride and 0.7 lb. Dowicide G for each 1,000 gal. of mash—for no attempt is made to sterilize it by steam. The mash is converted in a mash tub at 145°F. (62.7°C.) for 1 hr. with agitation. The mash that is to be converted by the molds is prepared in the same manner, except that the amount of malt is 2 per cent.

Addition of Inoculums.—The control mash (malt saccharified) is cooled, pumped into the fermenter and 4 per cent of yeast inoculum (starter), based on total charge, is added. The amount of mash and yeast added initially fills about 30 per cent of the fermenter. This mixture is aerated for 3 to 4 hr. Then the tank is filled to 20,000 gal. by the addition of two more mashes prepared as described above.

The procedure with the mold inoculum is similar, except that the contents of the 2,500-gal. vessel containing the mold are added to the fermenter along with the yeast. Conversion and fermentation proceed simultaneously.

Saccharification and Fermentation.—Saccharification and fermentation are complete in 40 hr. when the volume of mold inoculum represents 12 per cent of the final volume of the mash; and in 55 hr. when the volume of inoculum represents 6 per cent of the final volume of the mash.

Yields.—When mold was used as the principal conversion agent, the average yield from 10 fermenters was 12.1 proof gal. per 100 lb. of dry grain, which represented 91.2 per cent fermentation efficiency. When malt alone was employed, the average yield from 180 fermenters was 11.0 proof gal. per 100 lb. of dry grain, which represented 84 per cent fermentation efficiency.

ACID SACCHARIFICATION OF GRAINS AND OTHER STARCH-CONTAINING MATERIALS

Grains, vegetables, and other starch-containing materials may be saccharified through the use of acids, such as sulphuric or hydrochloric acids. In general, the products to be converted are macerated, or ground, mixed with a measured quantity of acidulated water, and treated with steam under pressure.

After saccharification, the mashes may be adjusted to the desired pH level for fermentation with ammonium hydroxide, sodium hydroxide, sodium carbonate, or calcium carbonate. The use of ammonium hydroxide is particularly desirable, since the ammonia may act as a source of nitrogen for yeast growth. If sulphuric acid is used for the conversion of the starch and subsequently neutralized with calcium carbonate, the precipitated calcium sulphate may be separated from the wort by sedimentation and/or filtration.

Severson[1] has investigated the saccharification of various grain mashes with hydrochloric acid. Maximum yields of ethanol from the acid-saccharified grains averaged somewhat lower than those obtained by malt saccharification. Highest yields of ethanol were obtained from corn when the mash was heated for 2 to 3 hr. at a steam pressure of 25 lb. per sq. in. in the presence of 0.10 N hydrochloric acid.[2]

TABLE 18.—EFFECT OF MALT, SOYBEAN MEAL, AND MOLD BRAN UPON THE ALCOHOL YIELDS FROM ACID-SACCHARIFIED CORN MASH[1]

Malt and soybean meal			Mold bran and malt			Mold bran and soybean meal		
Proportion of material as per cent of corn		Yield, per cent	Proportion of material as per cent of corn		Yield, per cent	Proportion of material as per cent of corn		Yield, per cent
M	S		MB	M		MB	S	
0	0	76.8	0	0	75.3	0	0	84.0
1	0	76.4	2	0	82.5	1	0	87.1
2	0	76.0	3	0	82.9	2	0	88.6
3	0	75.5	5	0	82.9	3	0	89.8
						5	0	92.4
0	2	75.7	0	2	78.0			
0	3	78.5	2	1	83.0	0	3	87.8
0	5	77.0	3	1	82.2	1	1	90.9
1	1	78.5	5	1	82.5	2	1	90.5
1	2	78.3				3	1	90.7
1	3	76.4	2	2	81.2			
2	1	76.5	3	2	82.6	1	2	90.7
2	2	77.5	5	2	82.7	2	2	91.2
2	3	76.6				3	2	91.3

[1] SCHOENE, L., E. I. FULMER, and L. A. UNDERKOFLER, *Ind. Eng. Chem.*, **32**: 544 (1940).

Schoene, Fulmer, and Underkofler[2] have shown that normal yields of ethanol may be obtained by the addition of mold bran to acid-saccharified mashes. Their procedure for preparing acid-saccharified mashes, based on the optimum conditions found by Severson, was as follows: Measured quantities of corn meal and 0.1 N hydrochloric acid, 100 g. and 500 ml., respectively, were placed in each 1,000-ml. Erlenmeyer flask used and the mixture was cooked for 2.5 hr. at a steam pressure of 25 lb. per sq. in. The pH of the cooked mashes was adjusted to 5.0 with concentrated

[1] SEVERSON, G., *Iowa State Coll. Jour. Sci.*, **11**: 215 (1937).

[2] SCHOENE, L., E. I. FULMER, and L. A. UNDERKOFLER, *Ind. Eng. Chem.*, **32**: 544 1940.

ammonium hydroxide. Weighed portions of amylolytic (enzyme-containing) materials were added to the acid-saccharified mashes and final saccharification was carried out at 55°C. for 60 min. Results of typical experiments by Schoene, Fulmer, and Underkofler are shown in Table 18 on previous page.

An examination of Table 18 indicates that the best results were obtained with mold bran. Schoene and his associates suggested that the increased yields resulting from the use of mold bran were due to the large variety of enzymes that it contained. For example, mold bran contains emulsin, an enzyme that may hydrolyze gentiobiose (a disaccharide that may be formed during acid saccharification) to dextrose.

Mixtures of mold bran and soybean meal also produced increased yields of ethanol from acid-saccharified corn mashes.

According to Hayek and Shriner,[1] starch-containing mashes may be hydrolyzed (saccharified) satisfactorily by the use of sulphurous acid. They showed that pure starch may be completely converted to glucose in 15 min. at 165°C. in the presence of 0.2 to 0.4 per cent of sulphur dioxide; that corn mash may be hydrolyzed in 15 min. at 160°C. with 2 per cent of sulphur dioxide; and that wheat mash may be hydrolyzed in 10 min. at 165 to 170°C. with 2 per cent of sulphur dioxide. As high as 95 per cent of glucose is obtained from corn and wheat mashes by hydrolysis with sulphur dioxide. The sulphur dioxide may be removed from the hydrolyzed mashes and fermentation initiated. Yields of ethyl alcohol obtained from sulphurous acid-hydrolyzed mashes compare favorably with those secured from malt-converted mashes, according to Hayek and Shriner.[1]

The pH of a mash saccharified with acid must be adjusted before it is inoculated with yeast or other fermentation organism. Such adjustment of the pH usually increases the salt concentration of the mash.

ACID SACCHARIFICATION OF AGRICULTURAL RESIDUES

Dunning and Lathrop,[2] of the Northern Regional Research Laboratory, have reported on their research concerning the laboratory-scale saccharification of agricultural residues, such as corncobs, cottonseed hulls, flax shives, oat hulls, and sugar-cane bagasse.

The agricultural residues, typical analyses for which are given in Table 19, are hydrolyzed in two stages. During the first stage the pentosans are hydrolyzed by the use of dilute sulphuric acid, and during the second stage the cellulose is hydrolyzed by the use of concentrated sulphuric acid.

[1] Hayek, M., and R. L. Shriner, *Ind. Eng. Chem.*, **36** (No. 11): 1001 (1944).
[2] Dunning, J. W., and E. C. Lathrop, *Ind. Eng. Chem.*, **37** (No. 1): 24–29 (1945).

TABLE 19.—PERCENTAGE ANALYSIS OF REPRESENTATIVE HARDWOODS, SOFTWOODS, AND AGRICULTURAL RESIDUES[1]

Material	Pentosan	Cellulose	Lignin
Ponderosa pine[2]	7.4	52.3	26.6
Tanbark oak[2]	19.6	44.8	24.8
Corncobs	28.1	36.5	10.4
Bagasse	20.4	41.3	14.9
Oat hulls	29.5	33.7	13.5
Cottonseed hulls	21.0	35.1	16.8
Flax shives	23.0	38.0	24.0

[1] DUNNING, J. W., and E. C. LATHROP, *Ind. Eng. Chem.*, **37** (No. 1): 24 (1945).
[2] RITTER, G. J., and L. E. FLECK, *Ind. Eng. Chem.*, **14**: 1050 (1922).

Pentosan Hydrolysis.—The purpose of this hydrolysis is to separate the pentosans as xylose and furfural without saccharifying the cellulose.

A study of the effects of acid concentration, temperature, time, and other factors upon the hydrolysis of pentosans has been made by Dunning and Lathrop[1] and it has been found that satisfactory hydrolysis may be accomplished under carefully controlled conditions. For example, when corncobs were treated with 1.9 per cent of sulphuric acid at a temperature of 121°C. for 50 min., using a solid to liquid ratio of 30:100, the yield of xylose was 86.1 per cent, that of furfural (as xylose) 9.3 per cent, and that of dextrose 0.78 per cent. Thus 95.4 per cent of the pentosans were hydrolyzed, whereas less than 1 per cent of dextrose was produced. Data concerning other conditions found satisfactory for the separation of pentosans from hexosans may be found in the table which follows:

TABLE 20.—EFFECTS OF ACID CONCENTRATION, TEMPERATURE, AND TIME UPON SEPARATION OF PENTOSANS FROM HEXOSANS[1]

Acid concentration (sulphuric), per cent	1.9	4.4	4.4
Temperature, °C	121	100	100
Time, min	50	55	50
Solids: liquid ratio, g./100 ml	30:100	6:100	6:100
1. Yield of xylose, per cent	86.1	84.1	86.9
2. Yield of furfural (as xylose), per cent	9.3	10.9	8.4
3. Sum of 1 and 2	95.4	95.0	95.3
4. Yield of dextrose, per cent	0.78	3.2	1.4

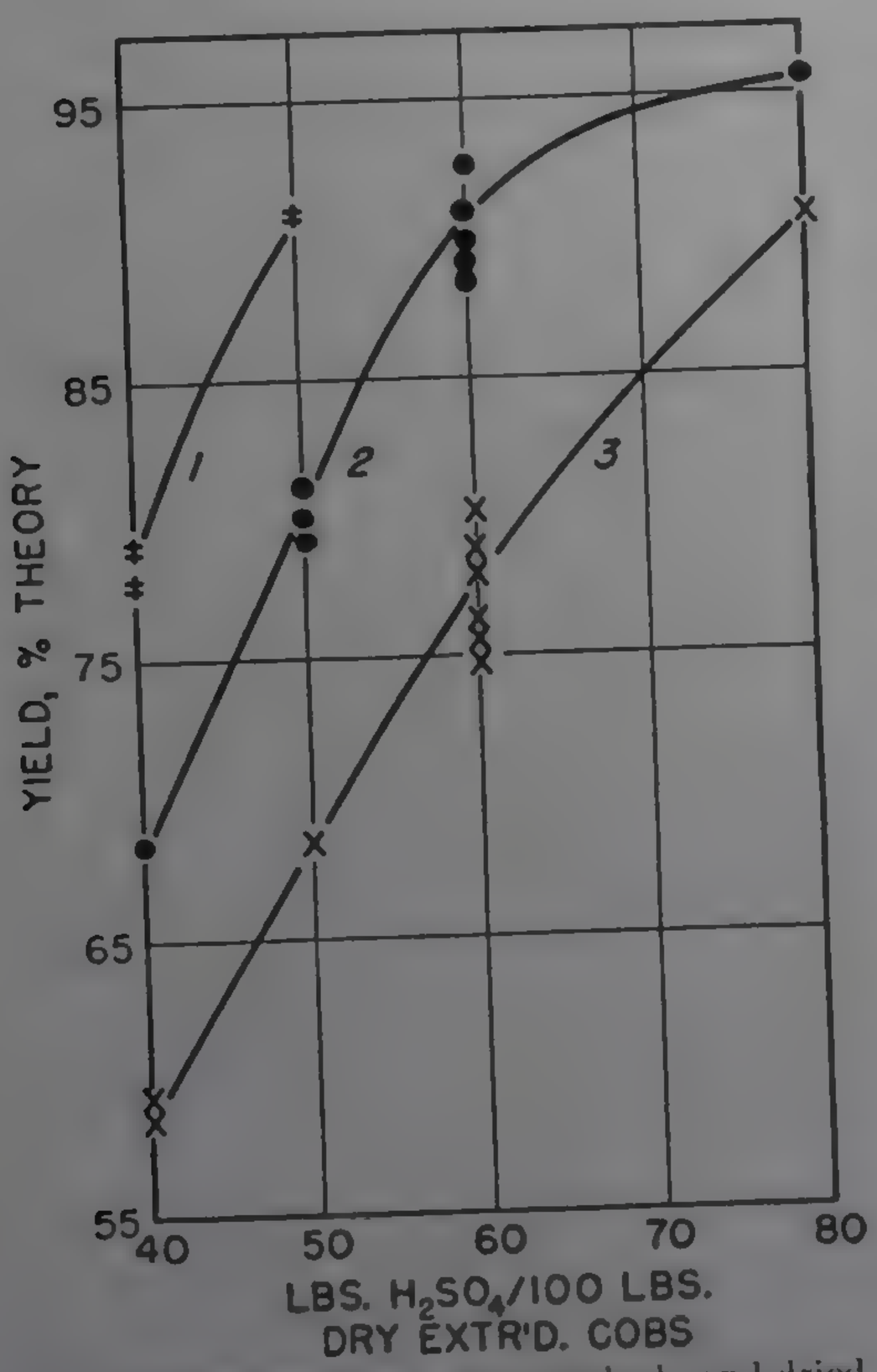
[1] DUNNING, J. W., and E. C. LATHROP, *Ind. Eng. Chem.*, **37** (No. 1): 24 (1945).

Experiments were carried out in a five-cell, glass diffusion battery on a laboratory scale to determine the value of countercurrent extraction of the pentosans from corncob residues. Each of the cells of the battery was

[1] DUNNING, J. W., and E. C. LATHROP *Ind. Eng. Chem.*, **37** (No. 1): 24–29 (1945).

equipped with the required inlets and outlets and a heating element. Dilute sulphuric acid at temperatures just under 100°C. was circulated through the crushed corncobs under a variety of conditions.

As a result of these experiments, certain facts become clear. Extraction of 90 to 95 per cent of the pentosans as hydrolyzate products could be accomplished consistently in 80 to 120 min. The hydrolyzates contained 14 to 20 per cent of reducing sugar. Sharp fractionation of pentosans from hexosans resulted only when conditions were limited. However, high yields of xylose could be obtained under a variety of conditions.



X = 8-mesh residue, water-washed, and dried.
● = 50-mesh residue, water-washed, and dried.
+ = 50-mesh with acid dried into residue.

FIG. 16.—Effect of variables on yield of dextrose from extracted corncobs impregnated with 85 per cent sulfuric acid at 40°C. [*Courtesy of J. W. Dunning and E. C. Lathrop, Ind. Eng. Chem.*, **37**: 24 (1945).]

Cellulose Hydrolysis.—The cellulose in the extracted corncobs is hydrolyzed in dilute acid solution after impregnation with concentrated sulphuric acid. One process developed by Dunning and Lathrop was essentially as follows:

The extracted corncobs were dried by filtration; by dewatering devices (to 50 to 55 per cent); and by the use of hot air at temperatures from 60 to 70°C., until the original acid from the pentosan hydrolysis was concentrated to 72 per cent. Residues dried in the presence of acid were more readily saccharified than those freed from acid before drying. The dried material was passed through a hammer mill to reduce it to the desired mesh size.

The dried residue was impregnated with sulphuric acid of 85 per cent concentration by means of a machine constructed of mild steel, which contained a hopper for feeding purposes, a discontinuous screw on a shaft cooled with water, and a water-jacketed casing. Figure 16 shows the effect of variables on the yield of dextrose from extracted corncobs impregnated with 85 per cent sulphuric acid at 40°C. Curve 1 of this figure shows that a 90 per cent yield of dextrose was obtained when 100 lb. of extracted corncobs (of 50-mesh size and with the acid dried into the residue) were treated with slightly under 50 lb. of 85 per cent sulphuric

acid at 40°C. for 2 min. According to the authors, this quantity of sulphuric acid is considerably less than that suggested in some other saccharification processes involving the use of concentrated sulphuric acid.

The residue, impregnated with 85 per cent sulphuric acid, occurring as a dry powder, is diluted with water and permitted to undergo hydrolysis. Dunning and Lathrop have reported that under optimum conditions—

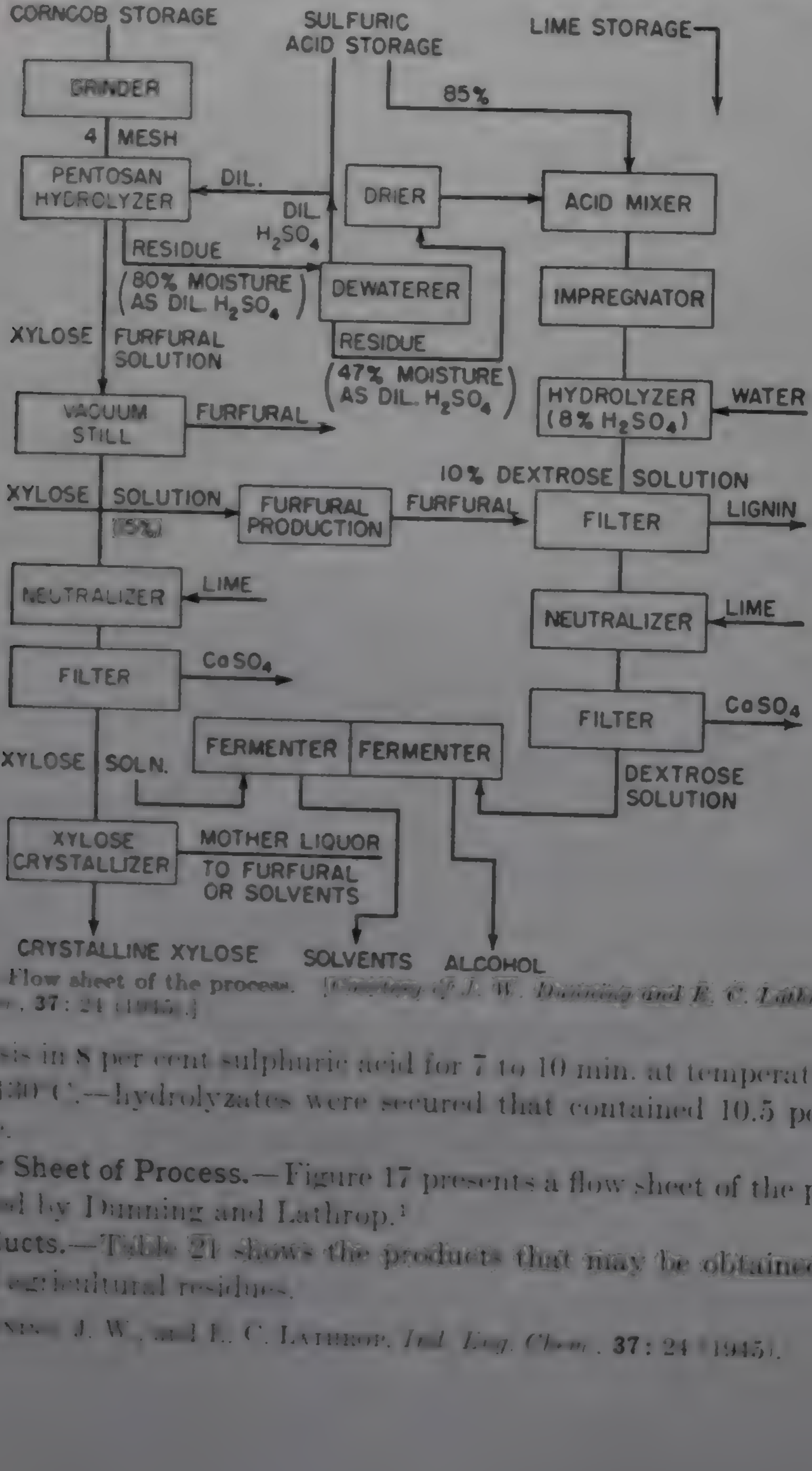


Fig. 17. Flow sheet of the process. [Courtesy of J. W. Dunning and E. C. Lathrop, Ind. Eng. Chem., 37: 24 (1945).]

hydrolysis in 8 per cent sulphuric acid for 7 to 10 min. at temperatures of 120 to 130°C.—hydrolyzates were secured that contained 10.5 per cent dextrose.

Flow Sheet of Process.—Figure 17 presents a flow sheet of the process developed by Dunning and Lathrop.[1]

Products.—Table 21 shows the products that may be obtained from 1 ton of agricultural residues.

[1] Dunning, J. W., and E. C. Lathrop, *Ind. Eng. Chem.*, **37**: 24 (1945).

TABLE 21.—PRODUCTS OBTAINABLE FROM ONE TON (DRY WEIGHT) OF AGRICULTURAL RESIDUES[1]

Agricultural residue	93 per cent pure crystalline xylose, lb.	Furfural, lb.	95 per cent alcohol, gal.	Lignin, lb.
Corncobs	135	214	44	227
Bagasse	98	155	49	327
Oat hulls	141	224	42	295
Cottonseed hulls	101	160	42	332
Flax shives	111	175	46	525

[1] DUNNING, J. W., and E. C. LATHROP, *Ind. Eng. Chem.*, **37**: 24 (1945).

Uses of the Hydrolyzates.—The dextrose solution obtained by the hydrolysis of cellulose may be used for the production of ethyl alcohol after the lignin has been removed by filtration and the excess acidity has been neutralized with lime.

The xylose solution resultant from the initial (pentosan) hydrolysis of the residues may be used for fermentation, for furfural manufacture, or for the production of crystalline xylose.

Prior to its use for fermentation purposes, it is necessary to neutralize the xylose solution (that obtained by the use of the countercurrent hydrolysis method containing approximately 15 per cent xylose, 0.3 per cent dextrose, 1.75 per cent furfural, 2.0 per cent acetic acid, and 4.4 per cent sulphuric acid) and to remove the furfural. The latter objective may be accomplished, according to Dunning and Lathrop, by feeding the hot xylose solution into a vacuum column and removing the furfural-water azeotrope. The xylose may be fermented to butanol and acetone by *Cl. acetobutylicum* (refer to the chapter on this fermentation) or to 2,3-butanediol by *A. aerogenes* (refer to the chapter concerning this subject).

ACID SACCHARIFICATION OF WOOD

For many years scientists have been engaged in developing and improving methods and equipment for hydrolyzing wood and other cellulose-containing substances to sugars. Their efforts have been rewarded with success, for there are several processes, some fundamentally different, by which sugars may now be prepared on a large scale from such materials.

1. Historical.—Dangiville, in 1880, proposed the use of gaseous hydrochloric acid to hydrolyze wood.

In Germany, Claassen (1900) developed a method for hydrolyzing vegetable fibers, using sulphur dioxide or sulphuric acid. Attempts to

establish commercial processes in the United States, based on his methods, resulted in failure.

In 1910, Ewen and Tomlinson[1] constructed the first plant for producing ethyl alcohol from sawmill waste in Georgetown, S. C. The average yield of alcohol, using dilute sulphuric acid under high pressure to hydrolyze the sawdust, was approximately 20 gal. per ton, although higher yields were occasionally obtained.

Willstätter (1913) demonstrated that a 40 per cent solution of hydrochloric acid was very much different in action toward cellulose than the common 35 to 36 per cent commercial hydrochloric acid solution. Willstätter, with Zechmeister, discovered that cellulose is transformed without waste to glucose within the space of a few hours by a 40 per cent hydrochloric acid solution at room temperature.

TABLE 22.—PRODUCTION OF ETHANOL FROM CELLULOSE, BY DIFFERENT PROCESSES[1]

Process	Year	Hydrolytic agent	Concentration, per cent	Temperature, degrees Centigrade	Liters of ethanol per 100 kg. of dried wood
Braconnot	1819	H_2SO_4	100		
Arnould	1854	H_2SO_4	100		
Simonsen	1894	H_2SO_4	0.5	180	7.6
Claassen	1900	SO_2	.....	150	7.5
Ewen and Tomlinson	1909	SO_2	.....	150	8.0
Cohoe	1912	HCl and vapor	.....	140	9.0
Willstätter	1913	HCl	40	20	
Hägglund	1914	H_2SO_4	0.4	175	8.8
Kressmann	1915	H_2SO_4	2.5	174	10.7
Meunier	1922	H_2SO_4	.....	170	15–20
Scholler	1929	H_2SO_4	0.5	180	22–24
Bergius	1931	HCl	40	20	32–35
Giordani-Leone	1939	H_2SO_4	.....	...	30

[1] GIORDANI, M., *Chimica e Industria* (*Italy*) **21**: 265 (1939).

Hägglund, Bergius, and others commenced work in 1916 which led to the development of the Rheinau process.

During World War I, the Germans produced sugars from wood by the so-called "Stettin war process." This process corresponded to the process used by Ewen and Tomlinson in the United States. From August, 1918, to September, 1919, the plant at Stettin[2] produced 300

[1] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr. Misc. Pub.* 327, December, 1938.

[2] SCHOLLER, H., *Chem. Ztg.*, **60**: 293 (1936).

tons of sugar and converted it to 150,000 liters of ethyl alcohol. Owing to the low yields, only about 6 liters of alcohol per 100 kg. of dry wood substance, it was economically unsound to continue operation of the plant after the war was over. It was later shown that the poor yields had been due to a partial destruction of the sugars during the hydrolytic process.

At Geneva, Terrisse and Lévy in 1920 used a combination of 40 per cent hydrochloric acid solution and gaseous hydrochloric acid to hydrolyze wood in a method that later became known as the Prodor process.[1]

Much research concerning the production of ethyl alcohol from wood wastes has been carried out by the U.S. Forest Products Laboratory at Madison, Wis. Sherrard and Kressman[2] reviewed the development of wood saccharification processes in the United States from the time of their inception up to prior to World War II. Harris, Beglinger, Hajny, and Sherrard[3] studied the hydrolysis of wood with dilute sulphuric acid in a stationary digester. They found that yields of about 50 per cent of reducing sugar (75 to 80 per cent fermentable) could be obtained from softwoods; and of about 50 per cent of reducing sugar (50 to 65 per cent fermentable) from hardwoods.

The use of the rotary digester in wood saccharification was investigated by Plow, Saeman, Turner, and Sherrard.[4] They reported that there were some serious disadvantages and little advantage in the use of the rotary digester in multistage saccharification of wood as compared with the vertical stationary percolator.

Saeman[5] studied the kinetics of the hydrolysis of wood and of the decomposition of sugars in dilute acid at high temperatures. He found that the hydrolysis of hemicellulose took place easily and required only a fraction of the time needed for the hydrolysis of the resistant portion of cellulose. Saccharification of cellulose involved two reactions: the hydrolysis to reducing sugar and the consecutive decomposition of the sugars. The decomposition of all the sugars appeared to follow the laws of a first-order reaction. An increase in acid concentration (0.4 to 1.6) or temperature (170 to 190°C.) resulted in increased efficiency in the formation of reducing sugar from cellulose.

An analytical procedure, stated to be rapid, was developed for the quantitative saccharification of wood and cellulose by Saeman, Bubl. and

[1] Ormandy, W. R., *Jour. Soc. Chem. Ind. (Trans.)*, **45**: 267T (1926).

[2] Sherrard, E. C., and F. W. Kressman, FPL Report No. R1454, Madison, Wis., September, 1944.

[3] Harris, E. E., E. Beglinger, G. J. Hajny, and E. C. Sherrard, *Ind. Eng. Chem.*, **37** (No. 1): 12 (1945).

[4] Plow, R. H., J. F. Saeman, H. D. Turner, and E. C. Sherrard, *Ind. Eng. Chem.*, **37** (No. 1): 36 (1945).

[5] Saeman, J. F., *Ind. Eng. Chem.*, **37** (No. 1): 43 (1945).

Harris.[1] The material for analysis is ground sufficiently finely to pass through a 30-mesh screen. It is treated with 72 per cent sulphuric acid at 30°C. for 45 min. after air-drying to a low moisture content. The mixture is diluted with water and treated for 1 hr. at a steam pressure of 15 lb., or by boiling for 4.5 hr.

Procedures for analyzing wood sugars have been described by Saeman, Harris, and Kline.[2] These included an electrometric titration method and a micro method developed by Shaffer and Somogyi for determining the reducing sugars; and a test for fermentable sugars.

Table 22 on page 99, summarizes some data concerning different methods for producing ethanol from cellulose.

2. The Bergius-Rheinau Process.—The Bergius[3] process is based on Willstätter's discovery that a 40 per cent solution of hydrochloric acid will hydrolyze cellulose to glucose at room temperature.

Cellulose is the principal constituent of wood. Hence a 40 per cent hydrochloric acid solution dissolves most of the wood except the lignin.

In the Bergius process, the wood is shredded and then dried in revolving drums to a water content of approximately 0.5 per cent. The dried wood is next conveyed to a battery of diffusers, where hydrochloric acid acts upon it to convert it to water-soluble sugars and other end products. A countercurrent principle is employed in the extraction. The acid progresses through the battery (Fig. 18) and comes into contact with fresh wood in the first diffuser. During the extraction process, about two-thirds of the wood by weight is dissolved, while one-third remains as lignin. The resultant acid solution contains approximately 32 per cent by volume of reducing sugar.[4]

The acid solution containing the reducing sugars (the hydrolyzate) is now distilled at 36°C. under vacuum in order to separate the bulk of the acid from the sugar. This procedure removes about 80 per cent of the acid from the hydrolyzate. The acid is regenerated, reconcentrated, and used over again.

The hydrolyzate is further concentrated in a spray-drying chamber where water and hydrochloric acid are lost by evaporation. The resultant solid product, which is voluminous, is collected in a cyclone, an apparatus that removes particles of dried material from the air by centrifugal force.

[1] Saeman, J. F., J. L. Bubl, and E. E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **17** (No. 1): 35 (1945).

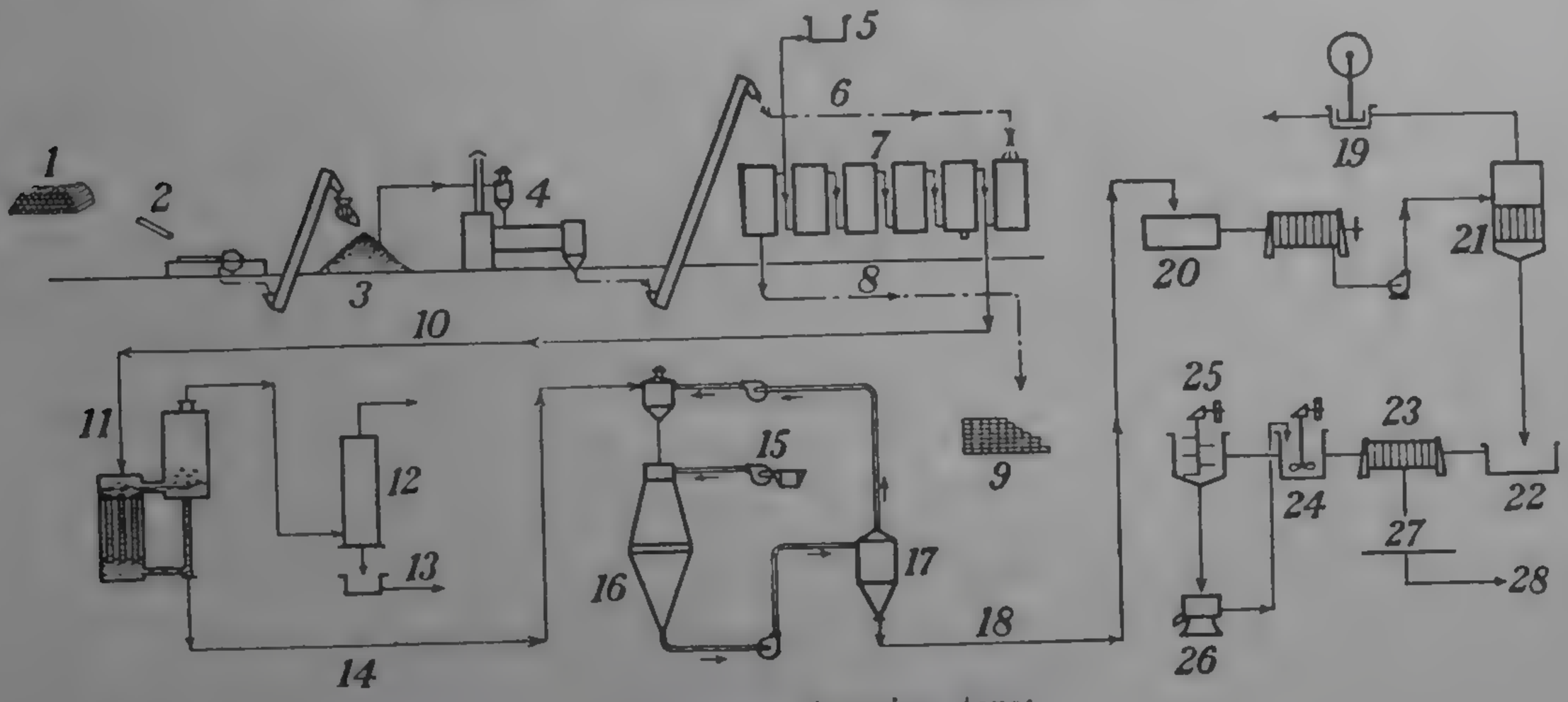
[2] Saeman, J. F., E. E. Harris, and A. A. Kline, *Ind. Eng. Chem., Anal. Ed.*, **17** (No. 2); 95 (1945).

[3] Bergius, F., *Ind. Eng. Chem.*, **29**: 247 (1937).

[4] *Ibid*

There are approximately 90 per cent sugars, 8 per cent water, and 1 to 2 per cent of hydrochloric acid in the solid hydrolyzate.

The sugars of the hydrolyzate, which are usually glucose, fructose, mannose, galactose, and xylose (depending on the nature of the wood hydrolyzed), are principally in a tetrameric form and are not directly fermentable. However, by diluting the solid hydrolyzate with 3 parts by volume of water and by heating the resulting solution at 120°C. for



Disintegration steps:
1. Wood supply
2. Wood shredder
3. Chips
4. Wood dryer
5. Hydrochloric acid
6. Dried wood chips
7. Diffusion battery
8. Lignin
9. Lignin-briquetting process

Evaporation steps:
10. Sugar solution
11. Evaporator for hydrochloric acid
12. Condenser
13. Hydrochloric acid
14. Sirup

Spraying steps:
15. Air heater
16. Spray dryer
17. Cyclone separator
18. Crude sugars (raw product)

Crystallization steps:
19. Vacuum pump
20. Inversion
21. Evaporator
22. First crystallization
23. Filter press
24. Solution vat
25. Recrystallization
26. Centrifuge for pure crystals
27. Sirup filtrate (mother liquor)
28. To alcohol (by fermentation)

FIG. 18.—Flow diagram of the Bergius process. [*Courtesy of Dr. F. Bergius, Ind. Eng. Chem.*, **29**: 247 (1937).]

0.5 hr., the tetrameric form is converted to a fermentable form. (Galactose and xylose are not fermentable by yeast.

According to Bergius, approximately 80 per cent of the raw sugar obtained by hydrolysis is fermented to alcohol, a long ton of dry wood yielding 85 to 90 gal. of ethyl alcohol (190 proof). The other 20 per cent of the sugar may be recovered and used for fodder or for other purposes.

The lignin, washed free of acid, may be used as fuel, or pure charcoal may be prepared from it.

Acetic acid, in quantities that correspond to those recovered during the destructive distillation of wood, is obtained as a by-product of this process.

3. The Scholler-Tornesch Process.[1]—In contrast to the Bergius process, the Scholler process employs dilute acid, elevated temperatures, and steam under pressure. The wood used does not have to be dried or of any definite particle size.[1] Furthermore, no attempt is made to recover the acid.

In 1926 and 1927 Scholler carried out research in the laboratory that indicated the value of the periodic removal of the sugar formed by pressure percolation. In Fig. 19 the curve X represents theoretical saccharification; the curve Z_1, the course of saccharification by the percolation method; and curve Z, the course of saccharification by a method that corresponds to the Stettin war process.

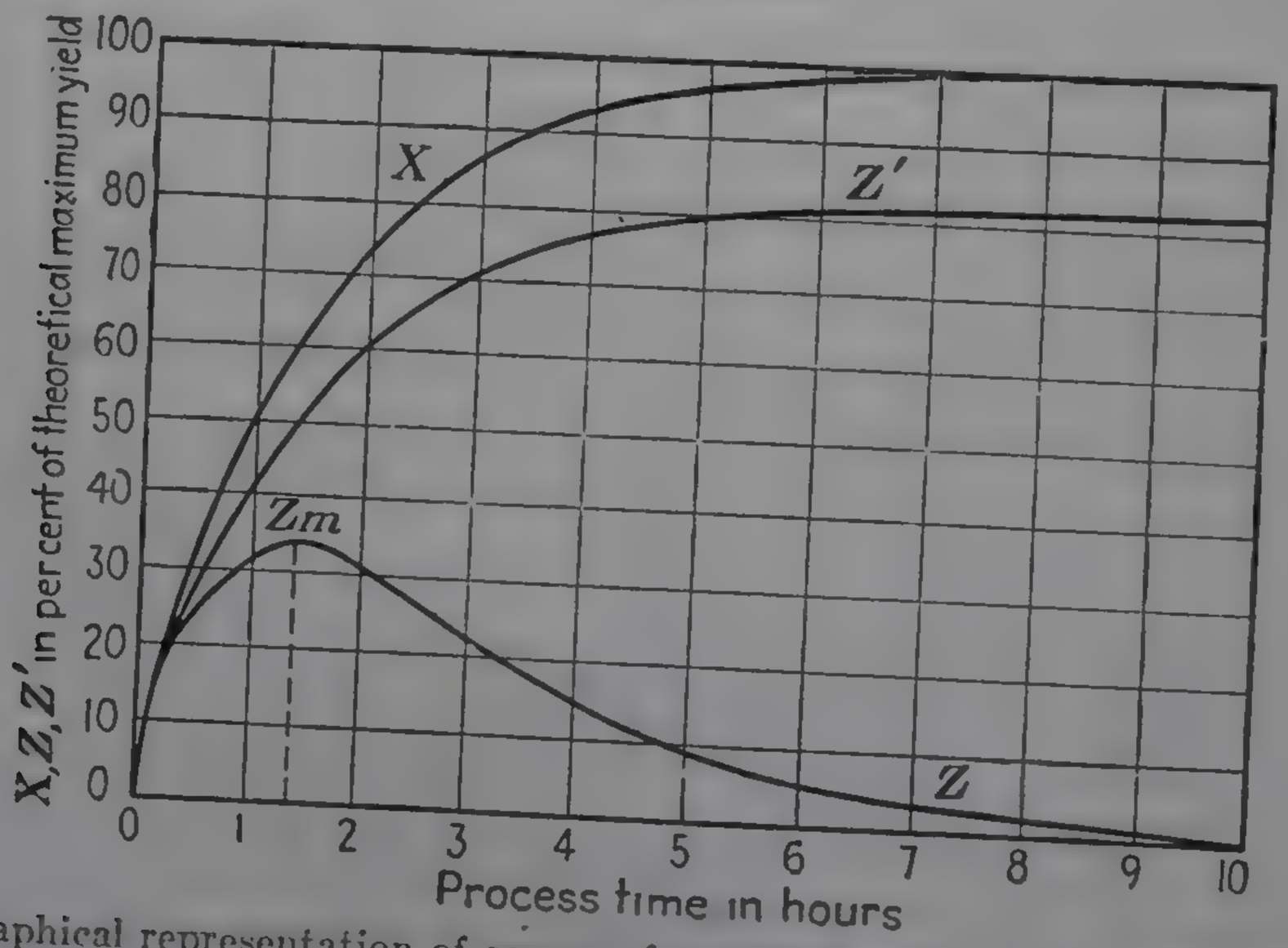


FIG. 19.—Graphical representation of course of saccharification in enclosed autoclave and percolator. [*Courtesy of Dr. H. Scholler, Chem. Ztg.*, **60**: 293 (1936).]

In the present industrial process, comminuted wood is carried by a system of conveyers to the top of a battery of percolators into which it is packed. Dilute acid solution is permitted to percolate intermittently under pressure through the heated wood, the accumulating sugar solution being removed periodically to prevent it from becoming destroyed. The sugar solution is neutralized with $CaCO_3$, permitted to settle in a tank, and filtered. It may then be fermented by yeast.

As stated above, the particle size of the wood is not of great importance. Sawdust, plane dust, rasp dust, and similar types of wood of variable moisture content may be used directly.

Each percolator is 14 m. in length and 2.4 m. in diameter with a capacity for 50 cu. m. of material. It is filled loosely with the cellulose-containing substance. After the percolator is closed, steam pressure is applied suddenly from above upon the loosely packed material, with the

[1] SCHOLLER, H., *Chem. Ztg.*, **60**: 293 (1936).

result that it is compressed evenly. The percolator is filled again and the material again compressed. The procedure is repeated until the percolator is almost completely filled with the compressed cellulose material.

The dilute acid solution used for hydrolysis is prepared by pumping sulphuric acid into water, which has previously been warmed by passage through a heat-exchange apparatus, to give an acid concentration of approximately 0.4 per cent.

This dilute acid flows through the cellulose-containing material at a temperature of about 170°C. and a pressure of 8 atmospheres. The sugar solution thus formed is removed shortly, passing through the heat exchanger where it gives off heat to water being warmed for the dilute acid solution. Hydrolytic action continues in the percolator after the removal of the sugar solution, owing to the moisture, acid, heat (160 to 190°C.), and pressure, but no additional sugar is removed until the next passage of the hydrolyzing solution.

After the sugars have been recovered from the wood waste by intermittent percolation with dilute acid, the lignin residue, which is in the form of a hard cake, must be removed from the percolator. Since lignin has a high water content and is at a temperature of about 180°C. at the end of the process, the sudden release of a section in the lower portion of the percolator causes an explosive expansion of the water in the lignin and the cake is shattered.

From the sugars obtained by the preceding method, industrially important alcohols, acids, or yeast may be prepared.

Ethyl alcohol yields amounting to 60 to 70 per cent of the theoretical are obtained by this process.

Lignin, acetic acid, furfural, and waste sugars are by-products.

TABLE 23.—PRODUCTS OBTAINED BY DIFFERENT PROCESSES FROM 100 KG. OF DRIED WOOD[1]

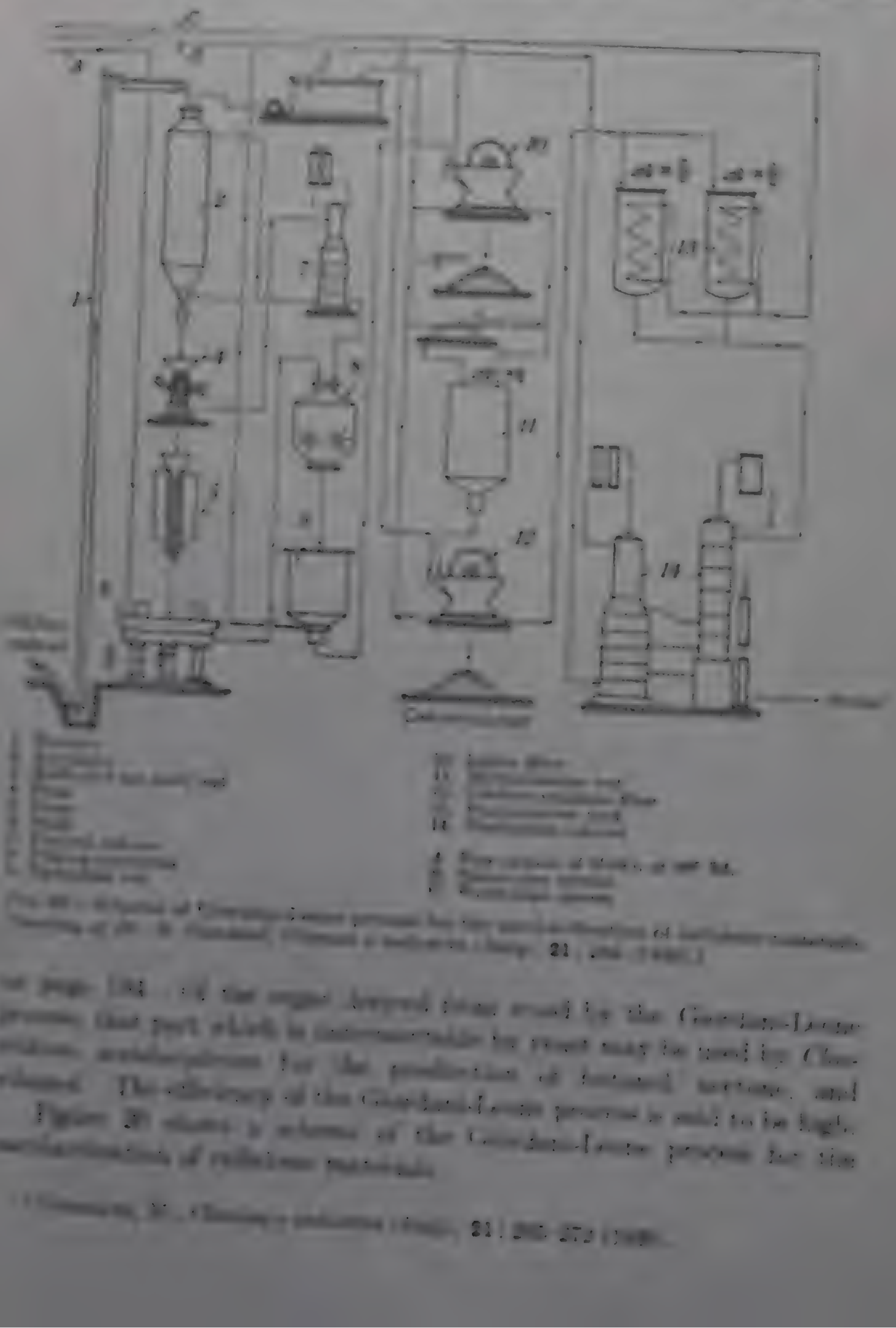
	Bergius, kg.	Scholler, kg.	Giordani-Leone, kg.
Acetic acid	4	[2]	0.2
Furfural	..	[2]	2
Lignin	33	30	30
Total sugars	66	43.3	60
Sugars fermented by yeast	56	35.2	48
Sugars not fermentable by yeast[3]	10	8.1	12
Yield of ethanol (liters of 100 per cent)	35	22	30

[1] Prepared from data presented by M. Giordani, *Chimica e industria (Italy)*, **21**: 265–272 (1939).

[2] Data not available.

[3] Approximate figures. May be utilized by bacteria in production of butanol, etc.

4. The Guichard-Lecuc Process. This process, described and discussed by Guichard,[1] makes use of 80% H_2SO_4. Space does not permit a detailed account of this process but some data concerning this, the Bergeim, and the Schaffer processes are presented in Table 23.



[1] Guichard, M., Chimie & industrie (Paris), 21, 1062 (1928).

See page 1063 of the original for a discussion of the Guichard process that part which is concerned with the use of the apparatus for the production of fumed sulfuric acid.

Figure 20 shows a schematic of the Guichard process proposed for the manufacture of sulfuric acid.

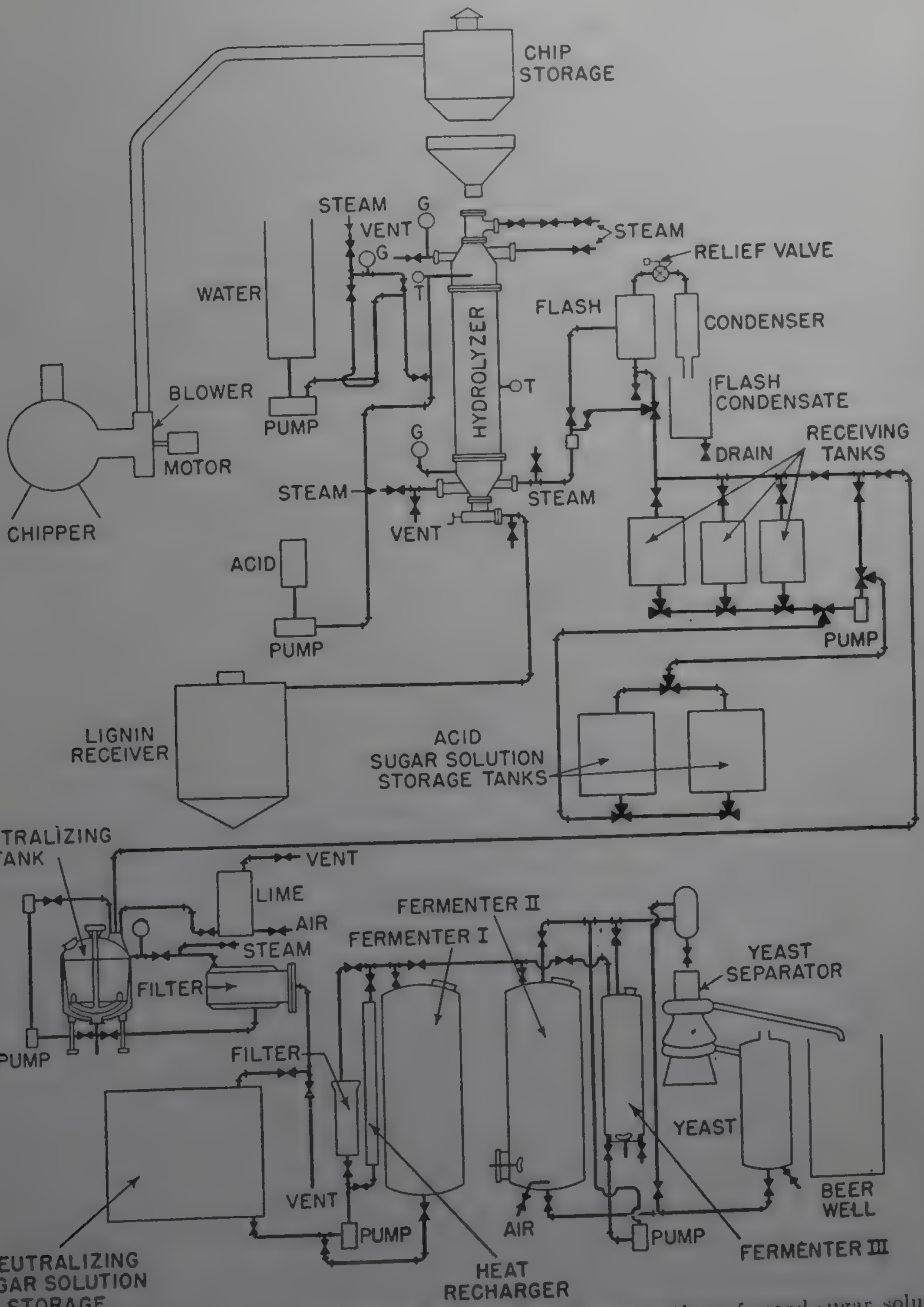


FIG. 21.--Equipment for saccharification of wood and fermentation of wood-sugar solutions. (*Courtesy of E. E. Harris and E. Beglinger, Mimeo. Report No. R-1617, Forest Products Laboratory, U. S. Dept. Agr., Madison, Wisconsin, June*, 1946.)

5. The Madison Wood-Sugar Process.[1]—This process was developed by the Forest Products Laboratory of the U.S. Department of Agriculture at Madison, Wis. Wood waste, consisting of edgings, chipped slabs, shavings, and sawdust, is hydrolyzed by permitting to flow continuously through it dilute sulphuric acid at temperatures of 150 to 185°C. Furfural and methanol are separated from the hydrolyzate by flashing. The hydrolyzate is then automatically neutralized with lime and filtered while under pressure. The solution of wood sugars may be used for purposes

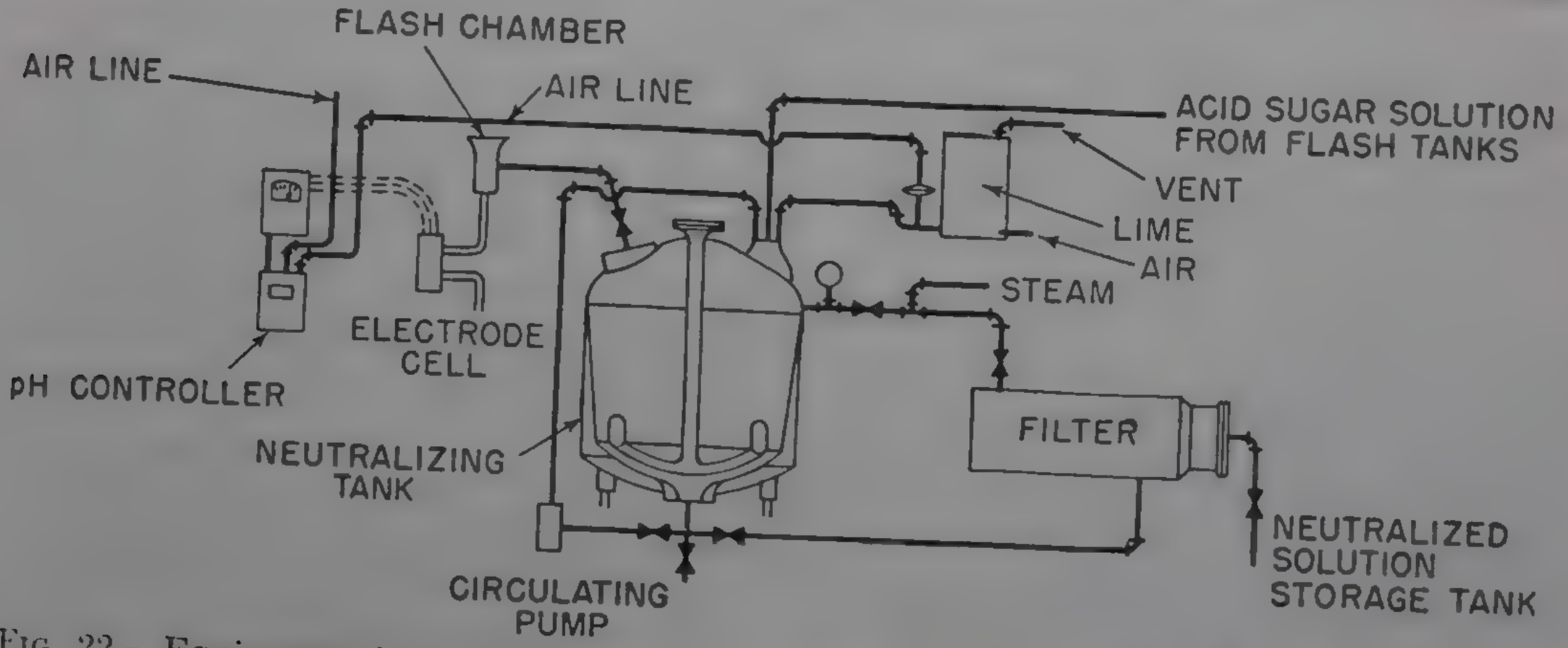


FIG. 22.—Equipment for neutralization of wood sugars. (*Courtesy of E. E. Harris and E. Beglinger, Mimeo. Report No. R-1617, Forest Products Laboratory, U.S. Dept. Agr., Madison, Wis., June,* 1946.)

such as the production of ethyl alcohol by fermentation with *Saccharomyces cerevisiae*.

The pilot-plant equipment for hydrolyzing wood wastes is shown in Figs. 21 and 22. It consists essentially of the following elements: a hog chipper used to break up pieces of wood waste to the desired size; facilities for lifting the waste to the storage chamber which is located directly above the digester; a digester constructed of silicon bronze having a capacity of 27 cu. ft. and of 23-in. inside diameter, with a reducing cone at the top and another plus a screen at the bottom; a water pump with a capacity variable from 2 to 10 gal. per min.; an acid pump with a capacity variable from 0.2 to 4 gal. per hr.; a switch for controlling the two pumps; a water heater with facilities for introducing steam into the water; a jet located in the water line for adding acid; two inlets at the top of the digester for introducing the dilute acid; a flash tank; receiving tanks with mixers; a neutralizing kettle; storage tanks; and a receiver for the lignin.

The wood waste used, Douglas fir, produces best results when present

[1] HARRIS, E. E., and E. BEGLINGER, *Report No. R-1617, Forest Products Laboratory*, Madison, Wis., June, 1946; also *Ind. Eng. Chem.*, **38** (No. 9): 890 (1946).

in the following percentages: 25 to 30 per cent shavings, 25 to 30 per cent sawdust, and 40 to 50 per cent edgings and slabs. These are hogged in such a manner that the pieces vary from 0.25 to 1 in. in length. Green wood waste containing 30 to 50 per cent of moisture is more satisfactory than dry wood because of the greater ease with which the acid diffuses through the material. Bark usually constitutes 35 to 50 per cent of the waste used. Yields of ethyl alcohol, used as an index of the efficiency of hydrolysis, are calculated on the basis of dry, bark-free wood. From 2 to 2.5 tons of green, bark-containing wood waste are generally necessary to yield a ton of bark-free wood on the dry basis.

During hydrolysis, best results are obtained when the digester is packed evenly with a uniform mixture of the hogged shavings, sawdust, edgings, and slabs. The digester is filled with the wood waste, the cover is put in place, and steam (at a pressure of 150 lb. per sq. in.) is introduced rapidly at the top (with vents open at the bottom of the digester) until the gauge at the top of the digester records a pressure of 10 lb. The steam is then turned off. When the gauge pressure has dropped to zero, the cover is opened and a new charge introduced, followed again by steam packing. After the packing is completed, steam is introduced through the bottom, with the top vents open, to remove more air and to continue the heating of the wood waste. Finally, the vents are closed and steam introduced to yield a pressure of 50 lb. in the digester.

Acid is introduced into the digester as follows: At the beginning, 200 lb. of sulphuric acid at a concentration of 1.5 to 2.0 per cent and at a temperature of 150°C. are added rapidly at the top of the digester. This amount of acid is required to bring the average acid concentration of the moisture present in the wood waste and added by the steam (about 600 lb. in all) to 0.5 to 0.6 per cent. Next, sufficient sulphuric acid (at a concentration of 0.5 to 0.6 per cent and at a temperature of 150°C.) is added at the rate of 12 gal. per min. to bring the ratio of dilute acid to wood (dry basis) to 3 to 1. This usually amounts to 400 to 600 lb. of dilute acid. Finally, 30 min. after the first acid has been added, the acid pump is started and dilute acid at a concentration of 0.5 to 0.6 per cent is added at the rate of 20 lb. per min. At the same time a temperature controller is set to cause the temperature in the digester to increase by 0.5°C. per min. for 30 min., or until a final temperature of 185°C. is obtained. This temperature is maintained until the end of the run.

Simultaneously with the addition of the dilute acid from the pump, the acid-sugar solution is removed continuously from the bottom of the digester at a rate of about 20 lb. per min. Thus the rate of addition of the acid to the digester and that of the removal of the hydrolyzate are the same.

The hydrolysis is continued until the average reducing-sugar concentration of the total combined hydrolysate is 5.0 per cent. This corresponds to a discontinuation of the hydrolysis after the concentration of reducing sugar in the hydrolysate has dropped to 0.5 per cent or less.

The hydrolysate is passed to a flash chamber where the pressure is dropped to 40 lb. per sq. in., permitting the steam to carry off the methanol and furfural. The latter are passed through a heat exchanger and, while being cooled, heat the water that is used in making the dilute acid.

The sugar solution is continually neutralized with lime and then heated to precipitate calcium sulphate while still at a pressure of 30 lb.

Finally, the sugar solution is cooled to 30° C. by flashing, and the precipitate which settles out is removed by filtration.

The usual time required for hydrolysis is 2.6 to 3.0 hr., although successfully it may be slightly shorter or longer.

A ton of dry, bark-free Douglas fir wood waste, when hydrolyzed by the foregoing process, yields 64.5 gal. of 95 per cent ethyl alcohol.

The advantages of this process as compared with the Scholler process are the shorter time required for hydrolysis; the lower steam requirement; the rapid removal of the sugars from the digester and the neutralization of the acid, which prevents their decomposition; the production of fewer substances inhibitory to fermentation; the longer life of the valves and other equipment, on account of fewer changes during operation; and the higher yields of sugars and of alcohol from the hydrolyzed wood waste.

References on Saccharifying Agents

Atkins, S. L., H. Rainwater, N. N. Anderson, and W. H. Stone: Submerged Culture of Fungal Amylase, *Ind. Eng. Chem.*, **39**: 1515–1517 (1947).

Bechtel, F. Rose: "Enzymes and Their Role in Wheat Technology," Interscience Publishers, Inc., New York, 1946.

Bibliography, List of Publications on Chemistry of Wood and Derived Products, *Report No. R1208, U.S.D.A., Forest Service, Forest Products Laboratory*, Madison, Wis., December 1943.

Bonnichsen, R., F. D. Snell jr., R. E. Scott, and L. A. Satina: Submerged Culture of Molds for Amylase Production, *Amer. Brewer*, **51**: 304 (1946).

Peterson, H., and I. M. Christensen: The Production of Ethyl Alcohol from Cull Potatoes and Other Farm Crops, *Bull. No. 241, Univ. Idaho Agr. Expt. Sta.*, Moscow, Idaho, 1941.

Pringsheim, P.: Conversion of Wood to Carbohydrates and Products in the Industrial Use of Concentrated Hydrochloric Acid, *Ind. Eng. Chem.*, **29**: 247 (1937).

Scott, R. E., and L. A. Satina-Rosenberg: Mold Bran Aids Production of Grain Alcohol, *Chem. & Met. Eng.*, **52** (No. 12): 110–111 (1945).

Thom, C., and M. L. Church: Method of Promoting Mold Growth. U. S. Patent 2,352,168, June 27, 1944.

Tollens, B.: Über Wirtschaftsgebiete der Holzverzuckerung von Holzzucker, *Chem. Ztg.*, **36**: 1305 (1912).

CORMAN, J., and A. F. LANGLYKKE: Action of Mold Enzymes in Starch Saccharification, *Cereal Chem.*, **25** (No. 3): 190–201 (1948).
DUNNING, J. W., and E. C. LATHROP: The Saccharification of Agricultural Residues, *Ind. Eng. Chem.*, **37** (No. 1): 24–29 (1945).
EFRON, A., and R. H. BLOM: Production of Maltose Sirups from Granular Wheat Flour, *Ind. Eng. Chem.*, **40** (No. 3): 412–415 (1948).
ERB, N. M., and F. M. HILDEBRANDT: Mold as an Adjunct to Malt in Grain Fermentation, *Ind. Eng. Chem.*, **38** (No. 8): 792–794 (1946).
———, R. T. WISTHOFF, and W. L. JACOBS: Factors Affecting the Production of Amylase by *Aspergillus niger*, Strain NRRL 337, when Grown in Submerged Culture, *Jour. Bact.* **55** (No. 6): 813–821 (1948).
FOTH, G.: "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.
GATES, R. L., and E. KNEEN: Preparation of Amylase-active Concentrates from Mold Bran, *Cereal Chem.*, **25** (No. 1): 1–19 (1948).
GIORDANI, M.: Alcool da materiali cellulosici, *Chimica e industria (Italy)*, **21**: 265–272 (1939).
GROVE, O.: The Amylo Process of Fermentation, *Jour. Inst. Brewing*, **20**: 248–261 (1914).
HAO, L. C., E. I. FULMER, and L. A. UNDERKOFLER: Fungal Amylases as Saccharifying Agents in the Alcoholic Fermentation of Corn, *Ind. Eng. Chem.*, **35**: 814–818 (1943).
———, and J. A. JUMP: Microbial Amylase Preparations—Conversion Agents for Alcoholic Fermentation, *Ind. Eng. Chem.*, **37**: 521–525 (1945).
HARRIS, E. E.: Saccharification of Wood, *Report No. R1475, Forest Products Laboratory*, Madison, Wis., March, 1945.
———, and E. BEGLINGER: Madison Wood-sugar Process, *Ind. Eng. Chem.*, **38** (No. 9): 890–895 (1946).
———, ———, G. J. HAJNY, and E. C. SHERRARD: Hydrolysis of Wood. Treatment with Sulfuric Acid in a Stationary Digester, *Ind. Eng. Chem.*, **37** (No. 1): 12–23 (1945).
HAWLEY, L. F., and L. E. WISE: "The Chemistry of Wood," Rheinhold Publishing Corporation, New York, 1926.
HAYEK, M., and R. L. SHRINER: Hydrolysis of Starch by Sulfurous Acid, *Ind. Eng. Chem.*, **36** (No. 11): 1001–1003 (1944).
JEFFREYS, G. A.: Mold Enzymes, Produced by Continuous Tray Method, *Food Inds.*, **20**: 688–690, 825, 826 (1948).
KATZEN, R., and D. F. OTHMER, Wood Hydrolysis—A Continuous Process, *Ind. Eng. Chem.*, **34** (No. 3): 314–322 (1942).
KRESSMAN, F. W.: The Manufacture of Ethyl Alcohol from Wood Waste, *U.S. Dept. Agr., Misc. Bull.* 983, 1922.
LE MENSE, E. H., J. CORMAN, J. M. VAN LANEN, and A. F. LANGLYKKE: The Production of Mold Amylases in Submerged Culture, *Jour. Bact.*, **54** (No. 2): 149–159 (1947).
———, V. E. SOHNS, J. CORMAN, R. H. BLOM, J. M. VAN LANEN, and A. F. LANGLYKKE: Grain Alcohol Fermentations, Submerged Mold Amylase as a Saccharifying Agent, *Ind. Eng. Chem.*, **41** (No. 1): 100–103 (1949)
LOCKE, E. G., J. F. SAEMAN, and G. K. DICKERMAN: The Production of Wood Sugar in Germany and Its Conversion to Yeast and Alcohol, *Fiat Final Report*, No. **499**, Nov. 14, 1945.

OLSEN, W. J., R. EVANS, and A. D. DICKSON: A Modification of the Kneen and Sandstedt Methods for the Determination of Alpha- and Beta- Amylases in Barley Malt, *Cereal Chem.* **21**: 533–539 (1944).

———, ———, and ———: A Corrected Unit of Alpha-Amylase Activity, *Cereal Chem.*, **24**: 299–300 (1947).

ORMANDY, W. R.: Sugar from Wood, *Jour. Chem. Ind. (Trans.)*, **45**: 267T (1926).

ORTVED, W. C.: The Application of *Taka-koji* in Distilleries, *Orig. Com. 8th Int. Cong. Appl. Chem.*, **26**: 69–76 (1912).

OWEN, W. L.: Production of Industrial Alcohol from Grain by the Amylo Process, *Ind. Eng. Chem.*, **25**: 87–89 (1933).

PLOW, R. H., J. F. SAEMAN, H. D. TURNER, and E. C. SHERRARD: The Rotary Digester in Wood Saccharification, *Ind. Eng. Chem.*, **37** (No. 1): 36–43 (1945).

ROBERTS, M., S. LAUFER, E. D. STEWART, and L. T. SALETAN: Saccharification of Wheat by Fungal Amylases for Alcohol Production, *Ind. Eng. Chem.*, **36**: 811–812 (1944).

ROSE, D.: The Amylase of *Bacillus polymyxa*, *Arch. Biochem.*, **16** (No. 3): 349–355 (1948).

SAEMAN, J. F.: Kinetics of Wood Saccharification—Hydrolysis of Cellulose and Decomposition of Sugars in Dilute Acid at High Temperatures, *Ind. Eng. Chem.*, **37** (No. 1), 43–52 (1945).

———, J. L. BUBL, and E. E. HARRIS: Quantitative Saccharification of Wood and Cellulose, *Ind. Eng. Chem., Anal. Ed.*, **17** (No. 1), 35–37 (1945).

———, E. E. HARRIS, and A. A. KLINE: Analysis of Wood Sugars, *Ind. Eng. Chem., Anal. Ed.*, **17** (No. 2): 95–99 (1945).

SANDSTEDT, R. M., E. KNEEN, and M. J. BLISH: A Standardized Wohlgemuth Procedure for Alpha-Amylase Activity, *Cereal Chem.*, **16**: 712–723 (1939).

SCHOENE, L., E. I. FULMER, and L. A. UNDERKOFLER: Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry, Comparison of General Saccharifying Agents, *Ind. Eng. Chem.*, **32**: 544–547 (1940).

SCHOLLER, H.: Die Gewinnung von Zucker, Spiritus und Futterhefe aus Holz als Rohstoff, *Chem. Ztg.* **60**: 293 (1936).

———: French Patents 706678; 777824; and 799358; U.S. Patents 1,641,771; 1,890,304; 1,990,097; 2,083,347; 2,083,348; 2,086,963; 2,088,977; 2,108,567; 2,123,211; 2,123,212; 2,188,192; and 2,188,193.

SEVERSON, G. M., and K. J. GOERING: Saccharification of Grain Mashes for the Alcoholic Fermentation Industry. Plant-scale Use of Mold Amylase, *Ind. Eng. Chem.*, **38**: 980–985 (1946).

SHERRARD, E. C., and F. W. KRESSMAN: A Review of Wood Saccharification Processes in the United States Prior to World War II, *Report No. R1454, Forest Products Laboratory*, Madison, Wis., September, 1944.

SOMOGYI, M.: A New Reagent for the Determination of Sugars, *Jour. Biol. Chem.*, **160**: 61–68 (1945).

STARK, W. H., S. L. ADAMS, R. E. SCALF, and P. KOLACHOV: Laboratory Cooking, Mashing, and Fermentation Procedures, *Ind. Eng. Chem., Anal. Ed.*, **15**: 443–446 (1943).

TAKAMINE, J.: Process for Producing Diastatic Product, U.S. Patent 1,054,324, 1913.

———: Diastatic Product, U.S. Patent, 1,054,626, 1913.

———: Enzymes of *Aspergillus oryzae* and the Application of Its Amyloclastic Enzyme to the Fermentation Industry, *Ind. Eng. Chem.*, **6**: 824–828 (1914).

———: Diastatic Product and the Process for Producing the Same, U.S. Patent 1,148,938, 1915.
———: Process for Producing Diastatic Product, U.S. Patent 1,263,817, 1918.
Thorne, C. B., R. L. Emerson, W. J. Olson, and W. H. Peterson: Evaluation of Malts for Production of Alcohol from Wheat, *Ind. Eng. Chem.*, **37**: 1142–1144 (1945).
Underkofler, L. A.: Production of Diastatic Material, U.S. Patent 2,291,009, 1942.
———: Microbial Amylases. Their Application to Alcoholic Fermentation, *Brewers Digest*, **17** (No. 11): 29 (1942).
——— and E. I. Fulmer: *Chronica Botan.*, **7**: 420 (1943).
———, ——— and L. Schoene: Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry. Use of Mold Amylase, *Ind. Eng. Chem.*, **31**: 734–738 (1939).
———, G. M. Severson, and K. J. Goering: Saccharification of Grain Mashes for Alcoholic Fermentation, *Ind. Eng. Chem.*, **38**: 980–985 (1946).
———, ———, ——— and L. M. Christensen: Commercial Production and Use of Mold Bran, *Cereal Chem.*, **24** (No. 1): 1–22 (1947).
Unger, E. D., H. F. Willkie, and H. C. Blankmeyer: The Development and Design of a Continuous Cooking and Mashing System for Cereal Grains, *Trans. Am. Inst. Chem. Engrs.*, **40**: 421–443 (1944).
Van Lanen, J. M., and R. H. Blom: "Report on Production and Utilization of Mold Bran," Northern Regional Research Laboratory, Peoria, 1945.
——— and E. H. Le Mense: The Production of Fungal Amylases in Submerged Culture and Their Use in the Production of Industrial Alcohol, *Jour. Bact.*, **51**: 595 (1946).
Vogel, E. H., F. H. Schwaiger, H. G. Leonhardt, and J. A. Merten: "The Practical Brewer," Master Brewers Association of America, St. Louis, Mo., 1947.
Willkie, H. F.: Distillery Processes, *Chem. & Met. Eng.*, May, 1945.
——— and P. J. Kolachov: "Food for Thought," Indiana Farm Bureau, Inc., Indianapolis, 1942.
Wohlgemuth, J.: Über eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments, *Biochem. Zeit.*, **9**: 1–9 (1908).

CHAPTER IV

THE PRODUCTION OF INDUSTRIAL ALCOHOL BY FERMENTATION

One of the most important and best-known industrial fermentations is that in which ethyl alcohol is produced from sugars by yeasts. The chemical manufacturer, the brewer, the distiller, the baker, the vinegar manufacturer, the scientist, the housewife, and many others depend in one way or another on the ability of the yeast to convert sugars to alcohol, carbon dioxide, and other end products. Since low-priced and waste carbohydrate materials may be used in the manufacture of ethyl alcohol, the fermentation process has not only great present but also vast potential value.

The subject of ethyl alcohol production by fermentation has assumed new interest on account of attempts to find substitutes for gasoline. Blends of alcohol with gasoline, especially a blend containing 10 per cent ethyl alcohol, may be used satisfactorily in the present internal-combustion type of motor. Present-day demands for motor fuels are great, but the sources of petroleum are limited.

Definitions.—Ethyl alcohol (CH_3CH_2OH), or ethanol, may be referred to by other names. For example, the alcohol may be named to indicate the source of raw material from which it is manufactured or to indicate the general purpose for which it is to be used. Grain alcohols are alcohols made from grains, such as corn, wheat, or rice. The term "grain alcohol" is sometimes used to signify ethyl alcohol in contrast to methyl alcohol (CH_3OH), or methanol, which is manufactured by the destructive distillation of wood, by synthesis, or by other means. Molasses alcohol is alcohol produced from sugar-cane molasses. Industrial alcohol is ethyl alcohol used for industrial purposes. Under this term is included power, or fuel alcohol, *i.e.*, alcohol used in combination with gasoline or other motor fuels.

The units of measurement commonly used in reporting ethyl alcohol production are the proof gallon, wine gallon, tax gallon, and barrel. The standard proof gallon is a wine gallon containing 50 per cent by volume (100 proof) of ethyl alcohol. A wine gallon is equal to a standard United States gallon and contains 231 cu. in. A tax gallon is equivalent to a proof gallon for spirits of a concentration of 100 proof or more and is

equivalent to a wine gallon for spirits of a concentration less than 100 proof. A barrel contains 31 wine gallons.

Proof is twice the per cent in volume of ethyl alcohol. For example, 95 per cent ethyl alcohol by volume is 190 proof.

Production Statistics.—The quantity of ethyl alcohol produced by 11 leading states and by the United States during the fiscal years 1942–1946 is shown in Table 24.

TABLE 24.—ETHYL ALCOHOL PRODUCTION BY LEADING STATES DURING FISCAL YEARS 1942–1946[1,2]

(Production, proof gallons)

State	1942	1943	1944	1945	1946
Pennsylvania	101,768,211	84,702,184	146,992,133	165,501,630	62,641,608
Louisiana	89,499,307	106,675,571	120,559,576	140,248,183	73,155,800
West Virginia	50,147,368	50,555,545	43,461,248	43,304,054	45,428,545
New Jersey	46,417,013	31,096,102	52,446,671	50,054,772	22,620,682
Indiana	39,841,119	30,538,951	53,342,979	28,242,636	28,078,096
Maryland	35,030,299	39,709,182	49,069,064	32,510,956	24,509,586
Texas	17,357,641	20,409,056	22,999,439	20,623,120	25,596,244
Kentucky	2,421,565	21,617,016	35,555,383	22,527,183	379,884
Nebraska	.....	.....	4,265,932	40,933,582	22,988,357
Iowa	.....	1,509,427	11,337,349	27,779,223	7,827,807
California	8,577,061	12,191,672	16,939,304	10,612,619	7,828,146
Total (United States, including Hawaii and Puerto Rico)	424,804,091	447,786,568	636,575,216	683,431,544	353,524,384

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1943, 1944, 1945, and 1946.

[2] Includes production by redistillation.

Raw Materials.—Ethyl alcohol may be produced from any fermentable sugar by yeasts under suitable conditions. Since starches and certain other carbohydrates may be hydrolyzed to fermentable sugars by biological or chemical means, there are available many possible sources of sugar.

1. *Types.*—Raw materials may be classified into three principal types: (*a*) the saccharine materials, such as sugar cane, sugar beets, molasses, and fruit juices; (*b*) the starchy materials, which include the cereals (corn, malt, barley, oats, rye, wheat, rice, grain, sorghum, and the like), potatoes, sweet potatoes, Jerusalem artichokes (girasol), manioca meal, and other substances; and (*c*) cellulosic materials, such as wood and waste sulphite liquor.

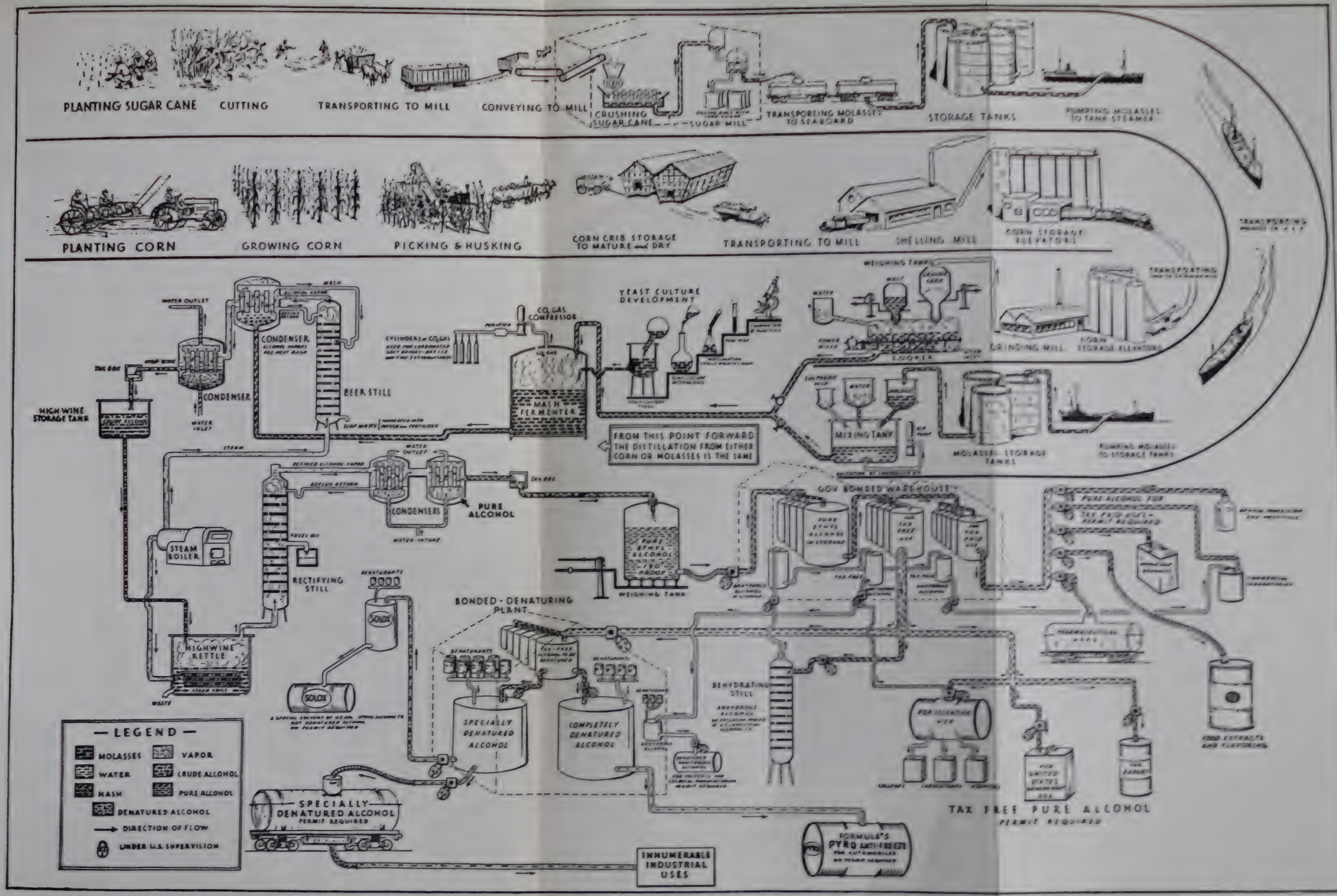


FIG. 23.—Flow sheet of industrial ethyl alcohol manufacture. *(Courtesy of the U.S. Industrial Chemicals, Inc., New York City.)*

2. *Principal Raw Materials Used.* In the United States, molasses and grain are the principal carbohydrate materials used in the production of ethyl alcohol. In Table 25 may be found the raw materials used for ethyl alcohol production during the fiscal years 1939, and 1941 to 1947.

Various types of raw materials are used in different countries. In Germany, for example, potatoes are used extensively; in France, sugar beets. Sweden manufactures much industrial alcohol from sulphite pulp. In Italy, maize beets, molasses, grapes, and other substances are used.

PROCESSES OF MANUFACTURE

The process used in the manufacture of ethyl alcohol by fermentation depends on the nature of the raw material. Saccharine materials usually require little or no special preliminary treatment other than dilution and may be fermented directly after certain customary adjustments have been made to the mash. But starchy and cellulosic materials must be hydrolyzed to fermentable sugars before they can be utilized by the yeast.

In each of the processes, success depends on the efficiency of preliminary treatment, if any; the use of an optimum concentration of sugar, an optimum pH, and an optimum temperature; the addition of nutrient substances to the mash, if it be deficient in any essential constituent; elimination of bacterial growth; the use of a vigorous strain of yeast with high alcoholic tolerance and an ability to produce large yields of alcohol; the maintenance of anaerobic conditions during the fermentation proper; and the prompt distillation of the fermented mash.

1. Ethyl Alcohol from Molasses.

a. Outline of Process.—The molasses mash is adjusted to the desired sugar concentration and temperature by the addition of water and to the desired pH by the addition of a measured quantity of acid. A yeast "starter" is mixed with the mash in the fermentation tank, which is usually covered, in one of several manners. Streams of the adjusted mash and the starter flowing simultaneously into the fermenter may be caused to converge on a baffle board located in the upper part of the tank. The mash and starter become well mixed as they splatter and fall to the bottom of the tank. An alternate method is to add the starter after the mash has been placed in the tank, and allow mixing of the two by mechanical agitation from time to time or by the bubbling of the tank. Another method would be to use paddles.

The fermentation rapidly becomes vigorous with the evolution of large quantities of carbon dioxide. In the modern plant, this gas is collected, purified, and used for the manufacture of dry ice or for other purposes. Within 30 hr, or less, the fermentation is usually complete. The fermented mixture, referred to as "beer," is distilled in a continuous unit to separate the alcohol and other volatile substances from the mash.

The alcohol is purified by means of rectifying columns and then stored in a bonded warehouse or denatured.

TABLE 25.—ETHYL ALCOHOL PRODUCTION, BY KINDS OF RAW MATERIALS USED, DURING THE FISCAL YEARS 1939 TO 1947[1]

(Thousands of proof gallons. Source: Bureau of Internal Revenue)

Raw material	1939	1941	1942	1943	1944	1945	1946	1947
Grain*	15,540	17,532	38,578	107,857	206,253	281,695	104,693	39,742
Molasses*	135,834	210,427	289,396	159,190	207,523	190,201	87,118	54,159
Ethyl sulphate	47,964	69,903	90,615	96,739	113,734	111,679	127,508	133,306
Unfinished spirits and other redistillation products	.....	.....	5,098	81,454	45,246	32,920	23,692	49,546
Sulphite liquors	.....	.....	.....	.....	.....	1,018	2,837	4,380
Pineapple juice	151	227	205	254*	438	241	250	220
Whey	.....	.....	.....	*	136	155	136	262
Crude alcohols mixture Cellulose pulp and chemical mixtures	310†	576	814	1,198	1,632	1,731	1,954	2,422
Potatoes*	.....	.....	.....	.....	224	.....	3,772	12,861
Other mixtures Grain, molasses, whey, pineapple juice, etc.	1,218	180	97	1,095	61,386	63,791	1,296	1,983
Total alcohol produced[2]	201,018	298,845	424,804	447,787	636,575	683,432	353,524	298,881

[1] HIBBEN, J. H., *Ind. Eng. Chem.*, **40** (No. 6): 990 (1948).
[2] Gross production; includes products used in redistillation.
* Additional amounts used in combination with other materials included under "other mixtures."
† Cellulose pulp included in "other mixtures."

b. The Process in Detail. (1) TYPE OF YEAST.—Certain types of yeasts are desirable, namely, those which are able to produce and tolerate high concentrations of alcohol and which possess uniform and stable characteristics. Strains of *Saccharomyces cerevisiae* are commonly used, but other yeasts, such as *S. anamensis* and *Schizosaccharomyces pombe*, may be employed under certain conditions.

(2) PREPARATION OF STARTER.—Having selected the yeast for the fermentation and having isolated it in pure culture, a starter is then prepared. A starter of large volume is required to "pitch" (inoculate) the main mash, which frequently may have a magnitude of several thousand gallons. Using aseptic technique, a tube containing about 10 cc. of sterile wort is inoculated from a pure culture of the yeast, which may be maintained on malt agar media. After incubation for a suitable period of time at a temperature of 25°C. (77°F.) to 30°C. (86°F.), the optimum for yeast growth, the culture in the tube may be used to inoculate a flask containing approximately 200 cc. of sterile mash. Following incubation, the contents of the flask may be used to seed a sterile mash of about 4-liter capacity. Up to this point in the preparation of the starter, the work is

ordinarily carried out in the laboratory, using glass containers. The next mash inoculated is of semi-plant-scale size (10 to 40 gal.) and is located close to the fermenters. Usually at least one more proportionally larger mash (several hundred gallons) is inoculated and permitted to incubate. Then this fermenting mash, the starter, is either pumped or allowed to flow by gravity to the main mash. The addition of this enormous yeast culture to the mash constitutes "pitching."

Aeration is of benefit in preparing a starter, for the object is to secure an immense number of yeast cells.

The Magné automatic system or other pure culture systems (Fig. 24) for preparing the starter may be used instead of the method just outlined. In the Magné apparatus a stock of the pure culture is maintained in the upper drum of the apparatus. Mashes are inoculated from this pure culture as required, and one culture may be used over a considerable period of time before recharging with a pure culture derived from a single cell. For further details, the reader may consult the reference given below.[1]

(3) THE MOLASSES.—"Blackstrap" molasses is the principal source of industrial alcohol. This material is the sirup that is left after the recovery of crystalline sugar from the concentrated juice of sugar cane. It usually contains 48 to 55 per cent of sugars, mainly sucrose. A large part of the blackstrap molasses used in this country is imported from Cuba, although some comes from Puerto Rico.

During recent years, much "high-test" molasses has been used for ethanol manufacture. This so-called "molasses" is an evaporated sugar-cane juice that contains all the original sugar of the juice, but most of it in an inverted form as a result of acid hydrolysis. Such molasses is usually high in sugar, containing occasionally as much as 78 per cent.

(4) CONCENTRATION OF SUGAR.—A sugar concentration of 10 to 18 per cent is usually satisfactory, although other concentrations are used. When the concentration is too high it reacts adversely on the yeast, or the alcohol produced may inhibit the action of the yeast, with the consequence that the fermentation time is prolonged and some of the sugar is not properly utilized. The use of too low a concentration of sugar is uneconomic as it may lead to a loss of valuable fermenting space. Furthermore a proportionally greater quantity of fermented wort must be distilled to produce the same amount of alcohol thus adding to the expense of production.

A sugar concentration of approximately 12 per cent is frequently used. The quantity of water required to reduce the molasses to the desired sugar content is calculated from available analytical data. For

[1] MAGNÉ, J. H. P., U.S. Patent 1,212,656, Jan. 16, 1917.

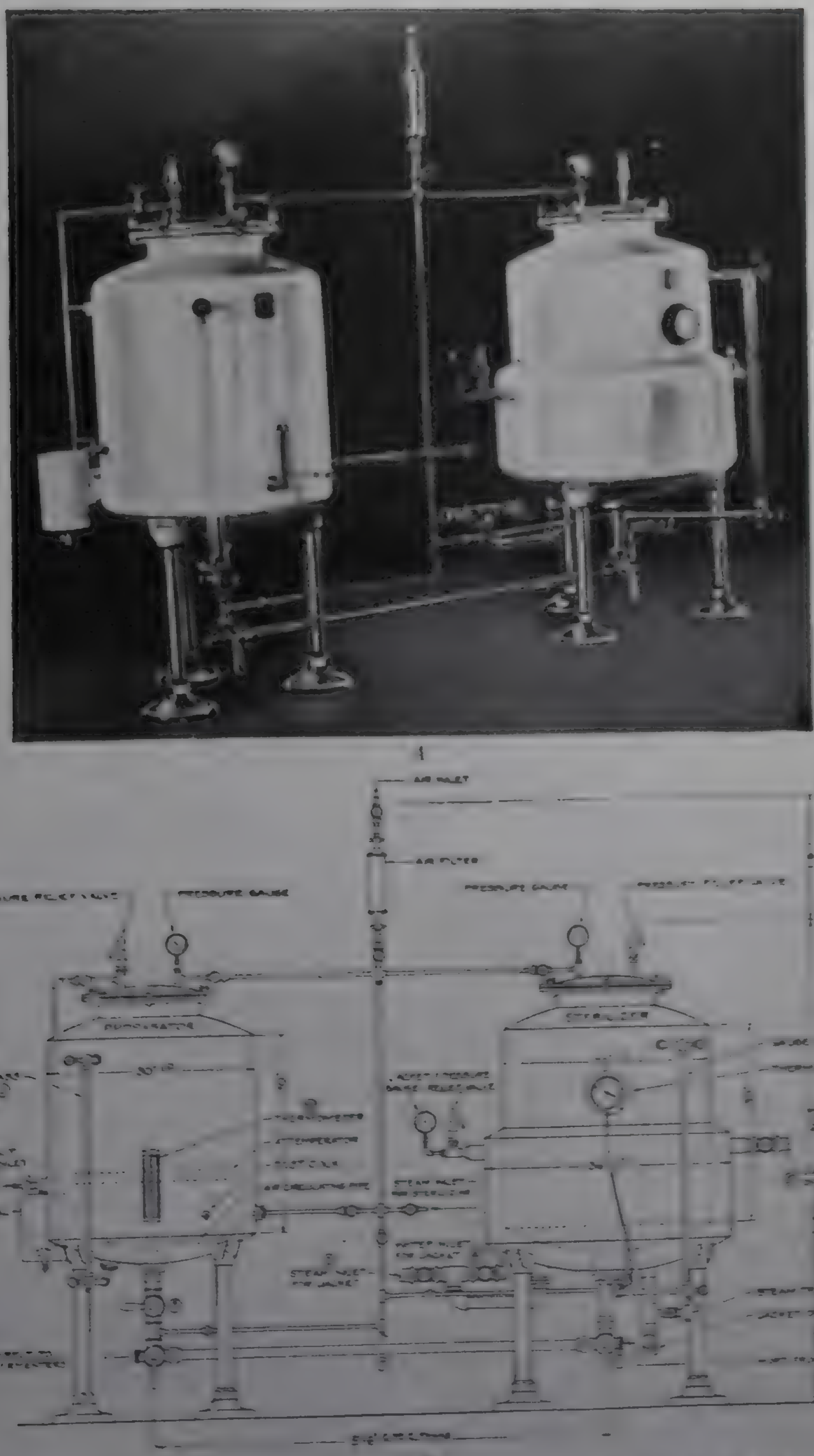


Fig. 24.—Pure yeast equipment. (*Courtesy of The Pfaudler Co., New York*)

example, 4 parts of water by weight would be added to 1 part of molasses, containing 60 per cent sugar, by weight, to reduce the sugar concentration to approximately 12 per cent.

The concentration of the sugar in a wort is usually determined by means of a Balling hydrometer, an instrument that gives the approximate percentage of sugar at 60°F., or 15.5°C.

(5) NUTRIENT SUBSTANCES.—Although molasses generally contains most of the nutrient substances required for fermentation, ammonium salts, such as ammonium sulphate or phosphates, may be added to the mash to supply deficiencies in nitrogen or phosphorus.

(6) pH OF THE MASH.—Fermentation proceeds satisfactorily when the mash has been adjusted to a pH of 4.0 to 4.5. This pH favors the yeast but is sufficiently low to inhibit the development of many types of bacteria. The manufacturer depends on the pH of the wort and the use of a large inoculum to take the place of sterilization, since it is costly and impracticable to sterilize large molasses mashes. Ordinarily the main mash is inoculated with a starter that represents 4 to 6 per cent of its volume, although the starter may represent from 2 to 25 per cent of the volume of the main mash under certain circumstances.

Sulphuric acid is commonly used to adjust the reaction of the mash, although lactic acid is satisfactory. Lactic acid favors the development of yeast but inhibits the growth of the butyric acid bacteria, which are detrimental to the yeast fermentation. As a substitute for the addition of acid, the mash may be inoculated with lactic acid bacteria, prior to the alcoholic fermentation.

(7) OXYGEN TENSION.—Oxygen in large amounts is necessary in the early stages for the optimum reproduction of yeast cells but is not required for the production of alcohol. During the fermentation, carbon dioxide is evolved and anaerobic conditions are soon established.

(8) TEMPERATURE.—The mash is pitched at a temperature of 60 to 80°F., usually 70 to 80°F., depending somewhat on the external temperature. During fermentation, the temperature of the mash rises. The use of cooling coils or sprays on the outside of the tank helps to maintain a suitable temperature. At temperatures much above 80°F., alcohol evaporates rather rapidly. Bacterial growth is also favored.

(9) TIME REQUIRED FOR FERMENTATION.—A fermentation is usually complete in 50 hr. or less, depending on the temperature, sugar concentration, and other factors.

(10) DISTILLATION.—The fermented mash ("beer") is distilled to separate the ethyl alcohol and fusel oil from the other constituents of the mash. In case there is a shortage of fermentors and the "beer" cannot all be distilled immediately, part of the "beer" is pumped to a storage tank, known as a "beer well," where it is held until it can be distilled.

During distillation, fractions containing different concentrations of alcohol ("wines") and slops are separated. The fractions containing approximately 60 to 90 per cent of ethyl alcohol are known as "high wines." These fractions are concentrated to 95 per cent ethanol by further distillation or fractionation. The fractions low in alcohol, the "low wines," are usually redistilled with new lots of "beer." The slops are ordinarily discarded but may be used in a number of ways. Sometimes the slops may be used as a substitute for some of the water in diluting molasses for a new mash. The solids from slops may be concentrated by heat treatment and sold as a fertilizer constituent, for they contain potassium and phosphates in addition to other components. The slops may be used as a core binder (in foundries) or as an adhesive for artificial stone (briquette).

(11) YIELD.—The common yield from blackstrap molasses amounts to approximately 90 per cent of the theoretical, on the basis of the fermentable sugars.

(12) FINAL TREATMENT.—The 190 proof ethyl alcohol (95 per cent by volume) may be further purified, dehydrated, or denatured as prescribed by the Bureau of Internal Revenue. Thus it is possible to purchase 95 per cent ethyl alcohol, with or without denaturant; c.p. (chemically pure) ethyl alcohol of 96 per cent concentration; absolute alcohol, U.S.P. (United States Pharmacopoeia); and anhydrous denatured ethyl alcohol (water-free alcohol).

(13) FLOW SHEET.—Schemes for the production of ethyl alcohol from potatoes, corn, and molasses are shown on page 128.

c. Rapid Continuous Process.—Bilford and his associates[1,2] have described a laboratory process for producing ethyl alcohol from molasses by rapid continuous fermentation. The chief value of this process lies in the fact that considerably less equipment is required than for the conventional process.

The process was briefly as follows: A suitable fermentation medium containing 10 to 15 g. of reducing sugar per 100 ml. was prepared, inoculated with an appropriate concentration of yeast cells, placed in a fermentation vessel (refer to Fig. 25), agitated with carbon dioxide (1.8 to 5.4 liters per min. per liter of medium) or mechanically, and incubated at 32.2°C. During the fermentation the pH was maintained within the range of 4.5 to 5 by adjustment with ammonia at ½-hr. intervals and

[1] BILFORD, H. R., R. E. SCALF, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1406–1410 (1942).

[2] BILFORD, H. R., F. H. GALLAGHER, W. H. STARK, and P. J. KOLACHOV, pp. 173 209, "Food for Thought" (by H. F. Willkie and P. J. Kolachov), Indiana Farm Bureau, Inc., Indianapolis, 1942.

determinations were made usually at hourly intervals of the yeast count, the Balling degree, and the reducing-sugar content of the medium. After a stationary phase of 2.5 to 5 hr., continuous operation was begun in which fresh medium was fed into the fermentor at a given rate (varied from 5 to 35 per cent per hr.) and fermented medium was withdrawn at the same rate.

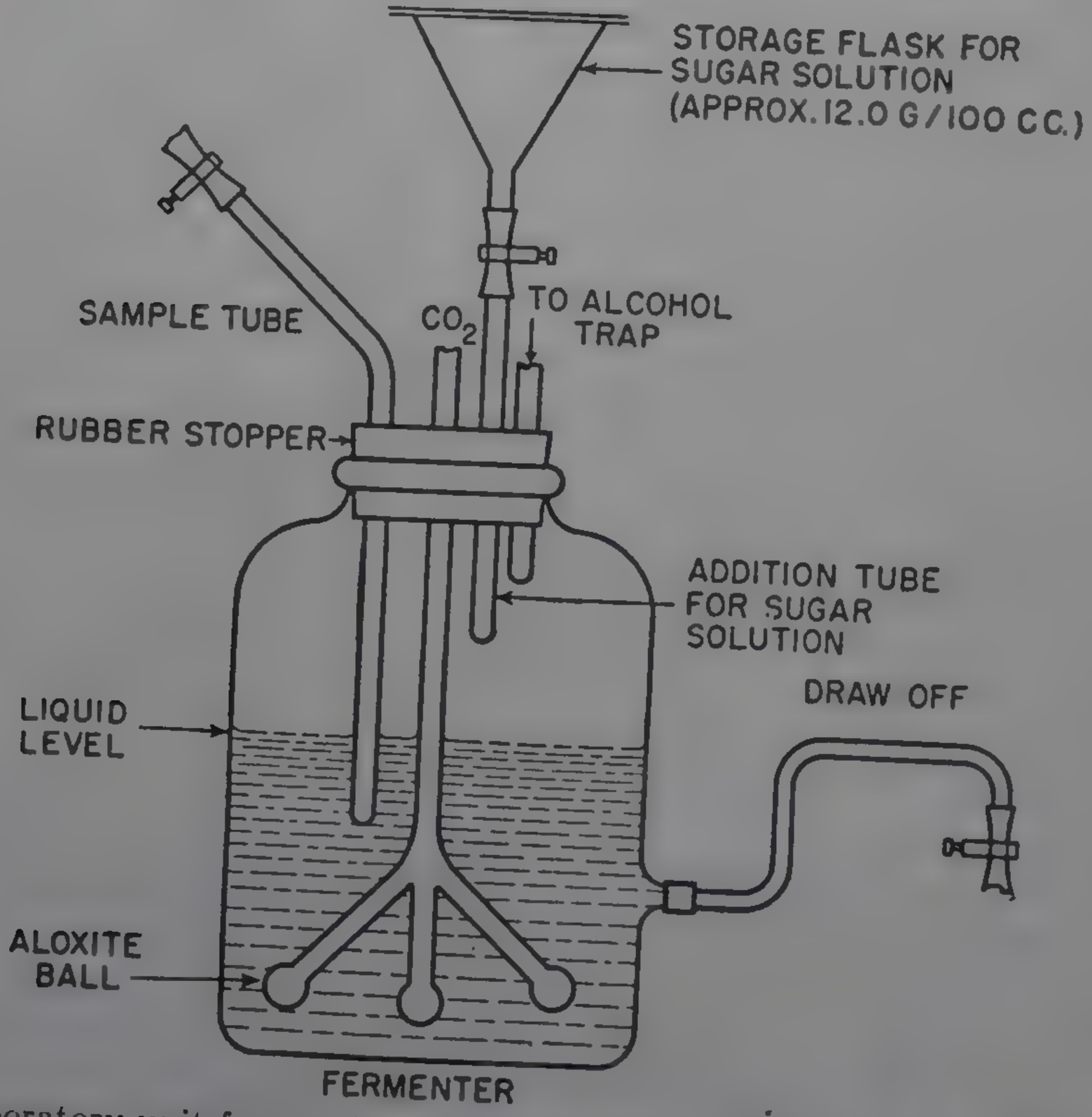


FIG. 25.—Laboratory unit for continuous fermentation. [*Courtesy of H. R. Bilford, R. E. Scalf, W. H. Stark, and P. J. Kolachov, Ind. Eng. Chem.*, **34**: 1406–1410 (1942).]

Bilford and his collaborators[1] experimented with a glucose-yeast water medium and with three molasses media. The glucose-yeast water medium was composed of 10 to 12 per cent of glucose dissolved in 10 per cent yeast water which contained 0.1 per cent of $(NH_4)_2HPO_4$. One of the molasses media contained Cuban blackstrap molasses with no supplement; the second, refined molasses plus 75 mg. of $(NH_4)_2SO_4$ per 100 g. of molasses; and the third, beet molasses plus 100 mg. of $(NH_4)_2HPO_4$ per 100 g. of molasses. Additional data concerning the molasses media may be obtained by reference to Table 26.

The yeast used for the fermentation of the glucose-yeast water medium was a variety of *Saccharomyces cerevisiae*, designated as Seagram No. 1. An inoculum of this yeast was prepared by growing it in an

[1] BILFORD, H. R., R. E. SCALF, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1406–1410 (1942).

TABLE 26.—RAPID CONTINUOUS FERMENTATION OF MOLASSES[1]

Hours	pH	3 N NH_4OH, ml.	Degrees Balling	Reducing sugar, g./100 ml.	Yeast, million cells/ml.	Throughput per cent total vol./hr.
A. Cuban Blackstrap, No Supplement						
0	4.92	...	18.0	12.02	...	Stationary phase
1	4.80	2	17.3	10.27	390	
2	4.75	2.5	15.4	8.15	...	
3	4.85	2	12.9	5.60	...	
4	4.80	3	9.8	2.56	550	
5	4.70	3	8.3	1.03	...	19
6	4.80	3	8.3	1.14	545	25
7	4.70	3	8.7	1.24	460	25
8	4.80	2	9.0	1.25	417	25
9	4.68	...	9.1	1.31	515	25
B. Refined Molasses + 75 Mg. $(NH_4)_2SO_4$/100 G.						
0	5.10	...	18.0	13.45	...	Stationary phase
1	4.80	3	17.1	9.87	315	
2	4.75	5	15.4	.....	...	
3	4.75	5	13.5	4.11	...	
4	4.56	8	11.4	.....	...	
5	4.80	5	9.3	3.95	515	15
6	4.80	5	7.2	1.69	...	15
7	4.60	7	6.4	1.12	580	20
8	4.70	6	6.5	.....	580	20
9	4.80	5	6.9	1.51	540	20
10	4.83	5	7.1	.....	508	20
C. Beet Molasses + 100 Mg. $(NH_4)_2HPO_4$/100 G.						
0	4.50	...	18.5	10.15	350	Stationary phase
1	4.45	...	15.5	8.81	400	
2	4.50	...	12.9	6.10	...	
3	4.45	...	10.2	3.77	...	
4	4.35	2	7.6	1.07	530	
5	4.50	...	6.8	0.53	.....	15
6	4.48	...	6.8	.....	...	15
7	4.50	...	7.2	0.85	380	15
8	4.40	1	7.4	0.62	...	15
9	4.52	...	7.2	0.87	360	15
10	4.50	...	7.2	0.76	360	15
10*	4.50	...	7.2	0.76	...	15
11*	4.50	...	6.9	0.76	375	15
12*	4.50	...	7.1	0.82	...	15

* Mechanical agitation.

[1] BILFORD, H. R., R. E. SCALF, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1406–1410 (1942).

aerated medium for 18 hr. at 30°C. The quantity of cells required for the fermentation was separated from the growth medium by centrifuging and was resuspended in 3,000 ml. of the glucose-yeast water medium. In one experiment, where the initial yeast count was 150,000,000 cells per ml., it required about 5 hr. to ferment 98.8 per cent of the sugar in a medium that before fermentation contained 10 g. of sugar per 100 ml. At this point the process was made continuous. By increasing the initial yeast count to about 350,000,000 cells per ml., it was possible to ferment 98.5 per cent of the sugar in 2.5 to 3 hr. under similar conditions, after which the processes were made continuous. It was possible thus to use a throughput rate of 25 per cent per hour, during which time the reducing sugar content varied between 0.1 and 0.4 g. per 100 ml. in the fermented medium and the yeast concentration remained fairly constant at the initial cell count. Refer to Table 27.

TABLE 27.—RAPID CONTINUOUS FERMENTATION OF GLUCOSE-YEAST WATER MEDIUM[1]

Hours	pH	3 *N* NH_4OH, ml.	Degrees Balling	Reducing sugar, g./100 ml.	Yeast, million cells/ml.	Throughput per cent total vol./hr.
0	5.30	..	10.6	10.52		
1	4.00	15	8.1	6.24	...	
2	4.20	10	4.0	2.39	355	Stationary phase
3	4.80	5	1.4	0.15	...	
4	4.65	5	1.4	0.20	365	25
5	4.70	3	1.2	0.25	350	25
6	4.70	3	1.1	0.32	357	25
7	4.70	3	1.0	0.22	351	25
8	4.80	3	1.0	0.37	327	25
9	4.75	3	1.0	0.13	334	25
10	4.70	3	1.0	0.11	321	25
					331	25

NOTE. Initial yeast count, approx. 350,000 cells/ml.; throughput rate constant.

[1] BILFORD, H. R., R. E. SCALF, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng. Chem.*, **34**: 1406–1410 (1942).

A different strain of yeast (*S. cerevisiae* Hansen (A.T.C.C. 4125) was used for the fermentation of the molasses media. The inocula for these media were prepared in a manner similar to that outlined above, except that molasses media were used for the production of the cells and that it was necessary to add 5 g. of malt sprouts per 100 g. of refined molasses in addition to the ammonium sulphate. Results of the experiments with the various molasses media are shown in Table 26.

The yields of ethyl alcohol obtained by the continuous process were comparable to those obtained by the conventional 50-hr. batch fermentation, according to Bilford and his coworkers.

For further details, refer to the original paper, also to the book entitled "Food for Thought," by Willkie and Kolachov.

2. Ethyl Alcohol from Whey.—A successful method for producing ethyl alcohol from whey has been developed by Browne[1] and by Rogosa, Browne, and Whittier[2] of the Division of Dairy Research Laboratories, Bureau of Dairy Industry, U.S. Department of Agriculture.

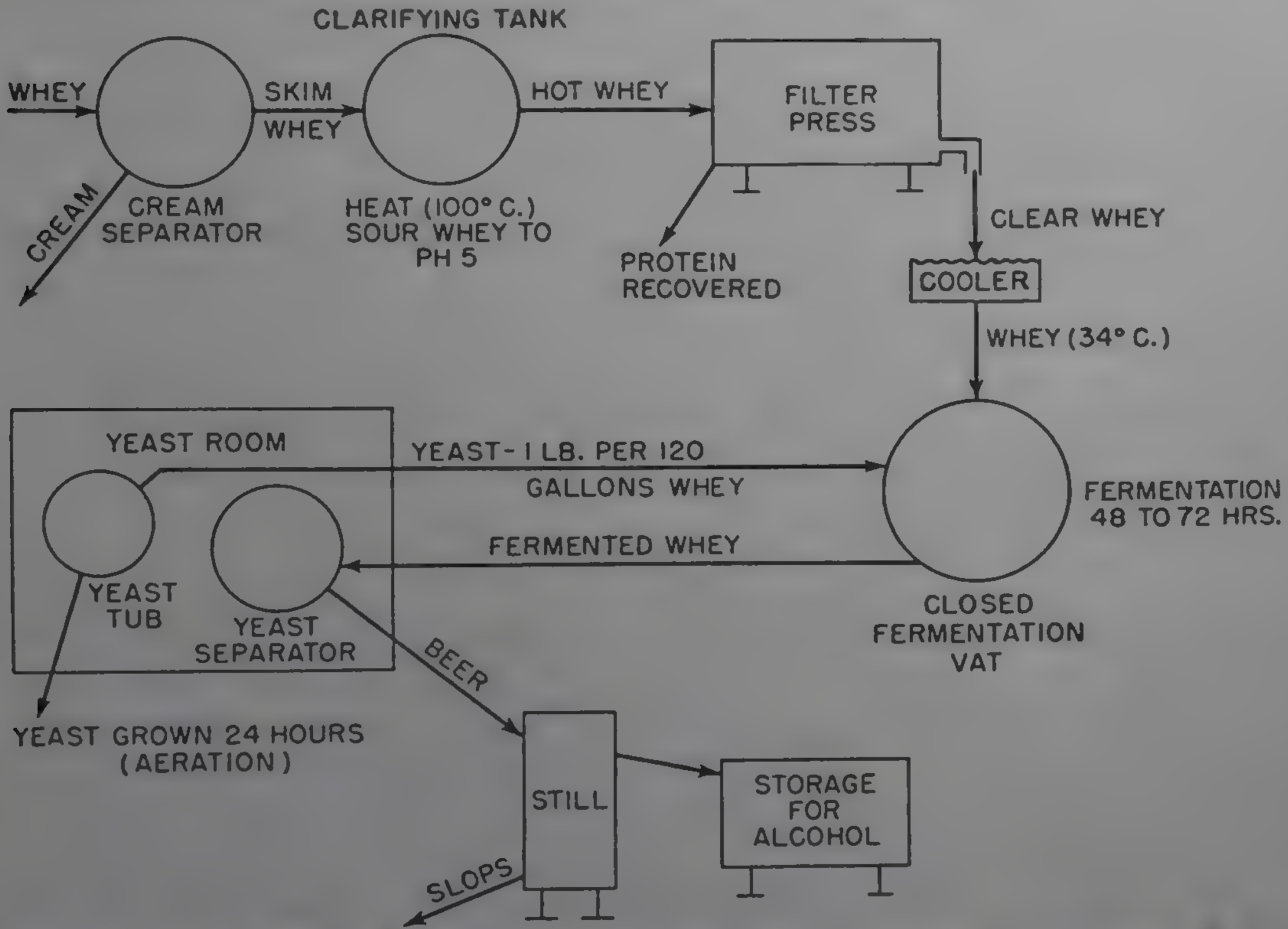


FIG. 26.—Flow sheet of alcohol production from whey. [*Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Science*, **30** (No. 4): 263 (1947).]

The process, in brief, consists of heating the whey to boiling, adjusting the pH to 5, separating out the protein by filtration, cooling the clear whey to 34°C. (93°F.), adding 1 lb. of *Torula cremoris* per 120 gal. of whey, carrying out fermentation at 33 to 34°C. for 48 to 72 hr., separating out the yeast, and distilling the alcohol. A flow sheet of this process is shown in Fig. 26.

Since the sugar of whey is lactose, it was necessary for Rogosa and his associates[2] to examine a number of lactose-fermenting yeasts to find one that would ferment all of the lactose in a relatively short time. Their findings, in respect to whey containing 5 per cent of lactose, are illus-

[1] BROWNE, H. H., *Ind. Eng. Chem., News Ed.*, **19**: 1271 (1941).

[2] ROGOSA, M., H. H. BROWNE, and E. O. WHITTIER, *Jour. Dairy Sci.*, **30** (No. 4): 263-269 (1947)

trated in Fig. 27. It is evident from this chart that *T. cremoris* was the most satisfactory yeast examined.

The maximum amount of yeast required for seeding was found to be 2 per cent of the weight of the lactose present in the whey before fermentation.

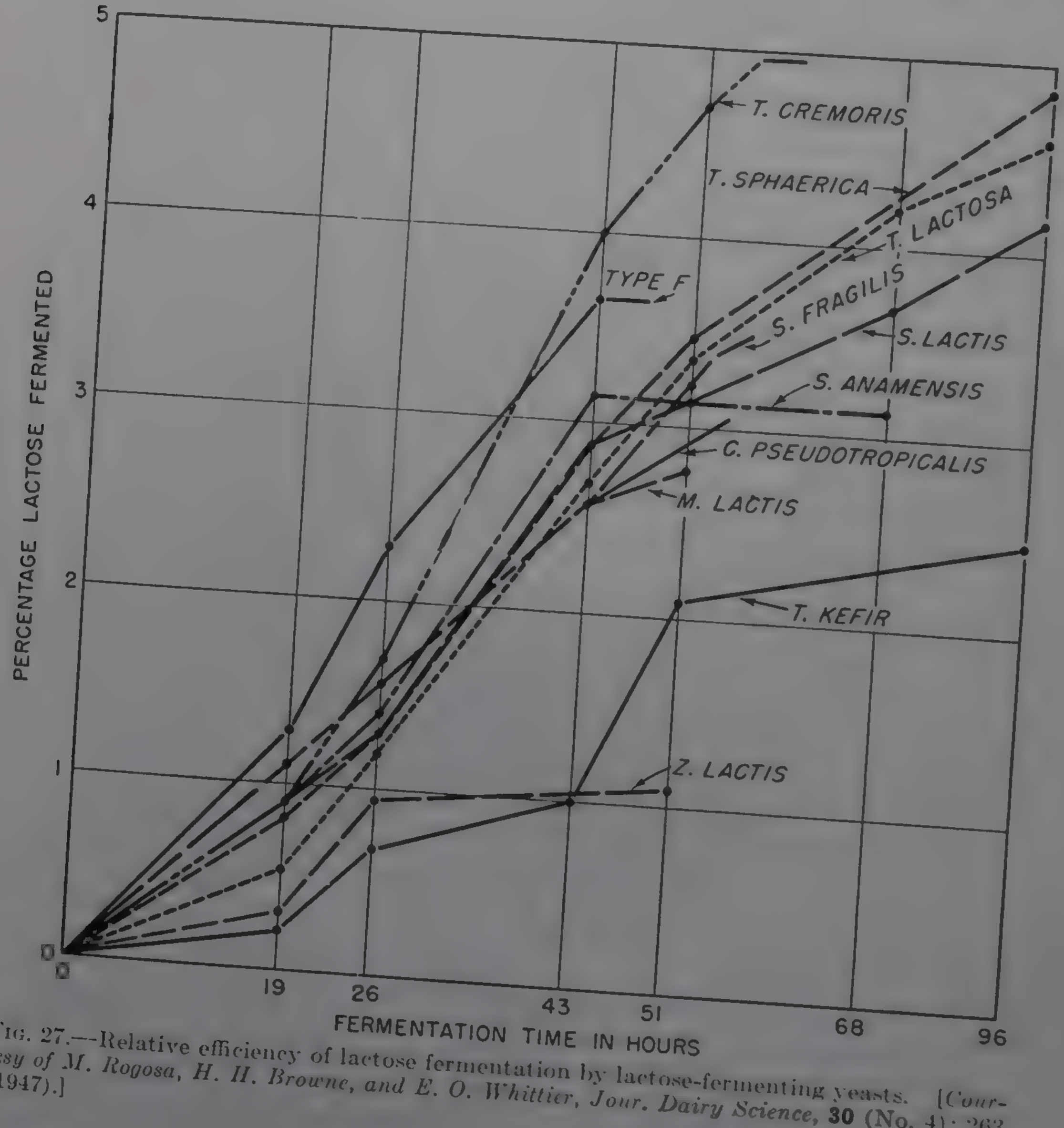


FIG. 27.—Relative efficiency of lactose fermentation by lactose-fermenting yeasts. [*Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Science,* **30** (No. 4): 263 (1947).]

The use of a temperature of 33 to 34°C. is recommended by Rogosa and his coworkers, although fermentation is actually more rapid at 37°C. than at any other temperature in the range of 30 to 42°C. However, the loss of alcohol through evaporation is more rapid also at 37°C. and the slight savings in time accomplished through the use of this temperature is not warranted. Likewise in the fermentation of 150-gal. batches of whey

started at 30°C. the temperature eventually reached a level of 33 to 34°C., at which level it remained during the most active part of the fermentation.

Clarification may be carried out by heating the whey, adding sour whey or acid, and filtering off the protein. The initial pH of the clarified whey should lie between 4.8 and 5.2.

Rogosa, Browne, and Whittier recommend that the pH of the whey

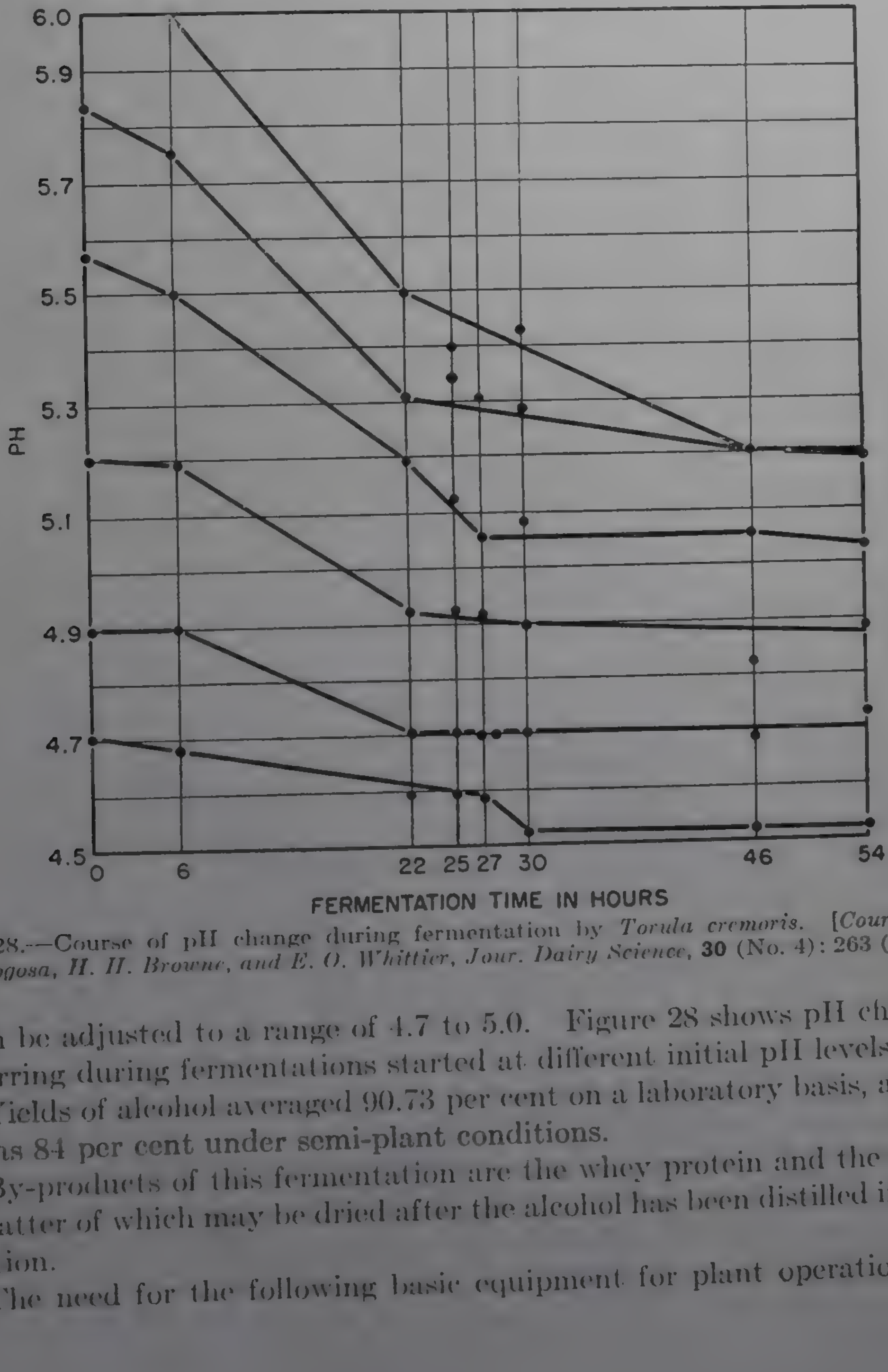


FIG. 28.—Course of pH change during fermentation by *Torula cremoris*. [*Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Science*, **30** (No. 4): 263 (1947).]

mash be adjusted to a range of 4.7 to 5.0. Figure 28 shows pH changes occurring during fermentations started at different initial pH levels.

Yields of alcohol averaged 90.73 per cent on a laboratory basis, and as low as 84 per cent under semi-plant conditions.

By-products of this fermentation are the whey protein and the slops, the latter of which may be dried after the alcohol has been distilled in acid solution.

The need for the following basic equipment for plant operation has

been indicated by Rogosa and his associates on the basis of their research with 150-gal. batches: a cream separator, a tank equipped with heat exchanger or liquid heater for heating the whey, a chemical feeder or proportioner for handling acid or sour whey, a filter press, a cooler, closed fermentation vats (fermenters), yeast tubs and a yeast room, an air line and filter for supplying sterile air, a yeast separator, a still, a storage tank for the alcohol, facilities for concentrating and drying the slops (if desired), pumps, pipe lines, and a steam supply.

A partial list of manufacturers of some of the foregoing equipment is supplied by the U.S. Department of Agriculture (BDIM-1011, May, 1947).

3. Ethyl Alcohol from Corn.—Normally corn is the chief grain source of ethyl alcohol in this country.

a. Preliminary Treatment.—Corn with or without the germs is ground, mixed with water, and cooked with steam under pressure to gelatinize the starch. The mash is then blown to a converter tub where additional water is added. After the temperature of the mash has been reduced to about 140°F. (60°C.), barley malt is mixed with the mash. The enzymes, contained in the malt, convert much of the starch of the corn to a fermentable sugar (maltose) and also break down some of the protein material. (Refer to the chapter on Brewing for details of the action of malt.) The corn may be saccharified by the use of mold bran, acid, or by another procedure (refer to Chap. III).

The mash containing the saccharified starch is transferred to a fermentor, adjusted to the proper sugar concentration by the addition of water, if necessary, cooled to a temperature of 65 to 80°F., and inoculated with yeast.

b. Flow Sheet.—A scheme for the production of ethanol from corn is shown in Fig. 29.

c. Some Special Uses.—Alcohol manufactured from corn is considered to be especially desirable for certain uses, on account of its freedom from foreign odors and flavors. For example, such alcohol may be used in the manufacture of perfumes, flavoring extracts, and high quality medicinals.

4. Ethyl Alcohol from Wheat.—Almost 700,000,000 gal. of alcohol were produced from wheat and its products during the period from late 1942 to July, 1945, according to Boruff and Van Lanen.[1] Wheat was used as a carbohydrate source because of the huge demand for alcohol, the shortage of molasses, and the surplus of wheat during the early years of World War II. However, the distillers were not experienced in the use of wheat and often did not have the best facilities for processing it or for

[1] Boruff, C. S., and J. M. Van Lanen, *Ind. Eng. Chem.*, **39**: 934 (1947).

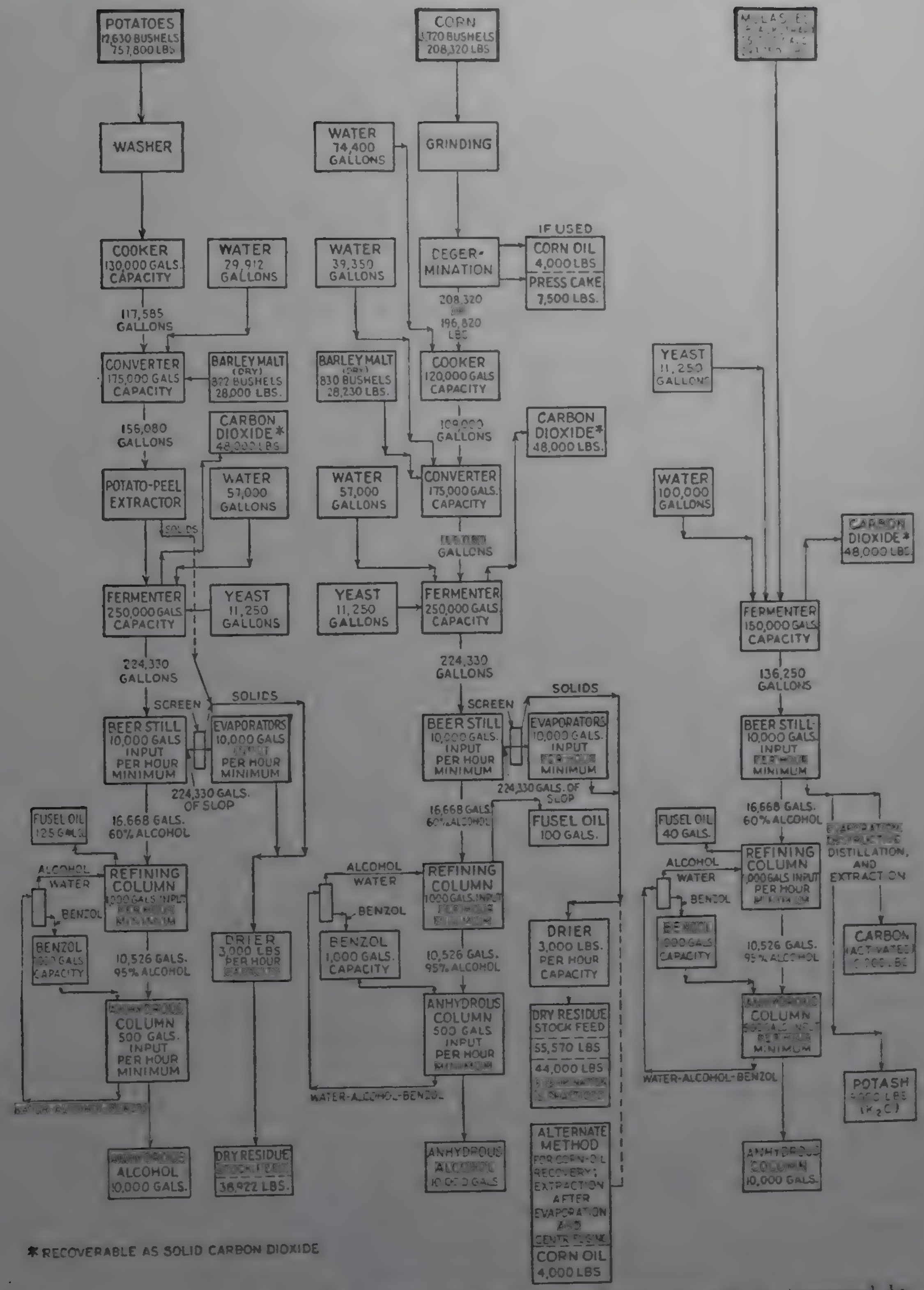


FIG. 29.—Conventional alcohol process. The residues, as shown, will be increased by the presence of yeast, yeast proteins, and unfermentable sugars. The figures actually used are based merely on the unfermentable residues calculated from the various analyses of the raw materials. (*Courtesy of P. Burke Jacobs and Harry P. Norton, Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Dept. Agr., Misc. Pub. 327, December*, 1938.)

recovering the by-products. Furthermore, there was considerable foaming during fermentation when the concentration of wheat was high.

A Wheat-Alcohol Research Committee was appointed by the War Production Board early in World War II to help solve the problems incumbent upon the utilization of wheat. The Northern Regional Research Laboratory was selected as the coordinating agency. A summary of conferences and collaborative research was published by the laboratory in March, 1944.[1]

Stark, Kolachov, and Willkie[2] reported their findings on the use of wheat for alcohol production. They found that the White or Soft Red Winter (Red Winter subclass) types of wheat were best suited for alcohol production; that Durum and Hard Red Spring wheats were generally not suitable because of their lower starch contents; and that Hard Red Winter wheat was intermediate between these groups in acceptability. They observed that pressure-cooking by the batch or continuous-process methods or atmospheric mashing at 155°F. were satisfactory.

The continuous pressure-cooking of wheat was carried out as follows: Soft Red Winter wheat (Red Winter Grade 1) was ground in such a manner that 55 per cent remained above a 20-mesh screen. The ground wheat was mixed with water and the premalt, precooked at 145°F. for 10 min., passed through a jet heater at 350°F. and held for 60 sec., then cooled to 152°F. (66.7°C.), in a continuous operation. In the atmospheric mashing process at 155°F., water (22 gal. per bu.) was heated to 110°F. and the wheat, ground as described above, was added. The mixture was heated to 155°F. in 45 min. and held at this temperature for 1 hr. Thereafter the mash was cooled to the conversion temperature (145 to 148°F.) and the conversion agents were added. A period of 30 min. was permitted for conversion.

The problem of foaming may usually be overcome by using mixtures of corn and wheat in which the wheat represents not more than 35 to 40 per cent of the mixture.

The recovery of distillers' grain and solubles was low early in the war due to lack of adequate facilities in the distilleries, but was high by 1945. Reports concerning by-products and their recovery have been made by Miller;[3] Jacobs;[4] Boruff;[5] and others.

5. Ethyl Alcohol from Dehydrated Sweet Potatoes.—The production of ethanol from dehydrated sweet potatoes of the Puerto Rico and L-4-5

[1] U.S. Dept. of Agriculture, Northern Regional Research Laboratory, March, 1944.

[2] Stark, W. H., P. Kolachov, and H. F. Willkie, *Ind. Eng. Chem.*, **35**: 133 (1943).

[3] Miller, E. S., *Am. Miller*, June, 1943.

[4] Jacobs, P. B., Northern Regional Research Laboratory, AIC-95 (July, 1946).

[5] Boruff, C. S., *Ind. Eng. Chem.*, **39**: 602 (1947).

varieties has been studied by Jump, Zarow, and Stark.[1] The dehydrated potatoes were in the form of sticks about $\frac{1}{8}$ to $\frac{1}{4}$ in. in diameter and up to $1\frac{1}{2}$ in. in length. The potatoes, ground or unground, were prepared for fermentation by mixing with stillage and/or water, by cooking, and by saccharification with barley malt. The mash bill used in the first fermentations was usually 89 per cent dehydrated sweet potatoes and 11 per cent barley malt. Previous to cooking (at 35 gal. of water and stillage per 56-lb. bushel of potatoes), the water and stillage, in a ratio of 2:1, were heated to 120°F. The potatoes, ground or unground, were then added together with 1 per cent of the malt for premalting purposes.

Atmospheric cooking at a pH of 5.3 was accomplished during the raising of temperature of the mash to 206°F. in 1 hr., the holding of the temperature between 206 and 202°F. for 1.5 hr., and cooling to 145°F. in 5 min. Pressure-cooking, when used, was accomplished in a similar manner, except that the mash was held at 206 to 202°F. for 0.5 hr. instead of 1.5 hr. and then was autoclaved for 1 hr. at 22 lb. of steam pressure.

Saccharification was initiated at 145°F. In the first fermentations half of the malt was added and the mash was held for 10 min. at 145°F. Then the remainder of the malt was added and the mash was cooled to the setting temperature. In an alternate method, all of the malt, except that used for premalting, was added and the mash was maintained at 145°F. for 60 min.

The stillage used was of two kinds: wheat-milo and sweet potato. During the first fermentations, sufficient stillage was added after the saccharification process to bring the concentration of stillage in the mash to 38 per cent.

A concentration of 45 gal. of mash per bushel of grain was used in setting the fermentors.

Table 28 shows alcohol yields from ground and unground dehydrated sweet potatoes.

Jump, Zarow, and Stark[2] concluded as follows as a result of their studies:

The L-4-5 variety was better than the Puerto Rico variety for alcohol production. Grinding was not necessary before cooking, although it increased yields slightly. Best yields of alcohol were obtained when the final mash contained 33.3 per cent by volume of a wheat-milo stillage. Yields were distinctly lower with sweet potato stillage or with no stillage. There were no significant differences in alcohol yields from mashes cooked by atmospheric or pressure methods. In a plant run, yields of 4.77 proof gallons of alcohol per bushel were obtained with Puerto Rico sweet potatoes, and 5.44 proof gallons per bushel with the L-4-5 variety. Cooking and conversion (saccharification) of dehydrated sweet potatoes

[1] JUMP, J. A., A. I. ZAROW, and W. H. STARK, *Ind. Eng. Chem.*, **36**: 1138 (1944).
[2] *Ibid.*

may be carried out successfully in grain distilleries without use of grain other than malt for conversion and without any change in the equipment.

TABLE 28.—ALCOHOL YIELD FROM DEHYDRATED SWEET POTATOES, GROUND AND UNGROUND[1,2]

Potato	Grind	Balling	Final data			
			Sugar, g./100 ml.	Alcohol yield, proof gal./bu.		Plant efficiency, per cent
				Wet	Dry	
L-4-5 (shredded)	Unground	0.6	0.72	6.06	6.50	92.5
	Coarse	0.6	0.80	6.16	6.61	94.1
	Fine	0.6	0.80	6.16	6.61	94.1
Puerto Rico (shredded)	Unground	1.7	0.78	5.76	6.27	86.6
	Coarse	1.9	0.96	5.70	6.32	87.3
	Fine	1.8	0.85	5.76	6.27	86.7
Puerto Rico	Unground	1.2	0.76	5.76	6.33	87.3
	Coarse	1.2	0.76	5.88	6.47	89.2
	Fine	1.2	0.75	5.89	6.48	89.3

[1] JUMP, J. A., A. I. ZAROW, and W. H. STARK, *Ind. Eng. Chem.*, **36**: 1138 (1944).
[2] Represents the average of three fermenters.

6. Ethyl Alcohol from Jerusalem Artichokes.—The Jerusalem artichoke, or girasol (*Helianthus tuberosus*), native to North America, is a plant that has been studied in recent years as a possible source of levulose and/or industrial alcohol. Although this plant is cultivated on a large scale in some parts of Europe, it is not an important crop in the United States at present, except in a few localities.

The Jerusalem artichoke is rich in the polysaccharide inulin $(C_6H_{10}O_5)_n$, which is readily hydrolyzed to levulose.

a. Yield per Acre.—The Jerusalem artichoke has been studied by Boswell and his associates[1] in considerable detail. Investigating 20 varieties of the Jerusalem artichoke, all grown in three different parts of the United States for three different years, they found that the mean yield per acre was 6.58 tons at Urbana, Ill., 16.73 tons at Corvallis, Ore., and 8.74 tons at Washington, D.C. The mean yield of the 20 varieties at all three places for three years was 10.69 tons per acre.

b. Sugar Content.—The sugar content of the tubers, after hydrolysis, varied in different seasons. A six-year mean analysis of the 20

[1] BOSWELL, V. R., C. E. STEINBAUER, M. F. BABB, W. L. BURLISON, W. H. ALDERMAN, and H. A. SCHOTH, *U.S. Dept. Agr., Tech. Bull.* 514, May, 1936.

varieties of Jerusalem artichokes investigated above showed 13.33 per cent levulose and 16.38 per cent total sugars.

c. Storage.—If an alcohol plant is to be operated continuously a constant supply of the raw material must be available. The fresh Jerusalem artichoke tuber cannot be stored satisfactorily. However, certain methods for storing the product have been worked out. McGlumphy and his associates[1] showed that the thinly sliced tuber could be satisfactorily desiccated and stored without sugar loss.

Both the dried and fresh tuber chips may be extracted by water in diffusion batteries, but such extracts deteriorate rapidly owing to microorganisms. If the extract, which contains the soluble sugars, is evaporated under reduced pressure to a total solid content of greater than 70 per cent, it becomes immune to decomposition by bacteria and yeasts. By storing the concentrated sirup under an atmosphere of carbon dioxide, mold growth is also prevented.[2]

The carbohydrates of the extract were stable at pH values between 4.8 and 9.0 at temperatures as high as 110°C.[2]

d. Fermentation.—For fermentation, the sirup is diluted to yield approximately 12 per cent reducing sugars after hydrolysis, sterilized, cooled, and inoculated with yeast. *Saccharomyces cerevisiae*, *S. anamensis*, and especially *S. pombe* have been used with satisfactory results by Underkofler and his associates.[2]

It is not necessary to make a preliminary hydrolysis of the sirup or to add additional nutrient substances.

e. Culture of Yeast.—When the yeast was continuously cultured on unhydrolyzed sirup from artichokes, its ability to produce ethanol was increased.[2]

f. Yields.—Yields of 90 per cent or more were obtained under the foregoing conditions.

7. Ethyl Alcohol from Sulphite Liquor.—Sulphite liquor is produced as a waste product in the manufacture of pulp from wood.

a. Pulping Process.—Spruce, hemlock, or some other kind of wood is cut into small chips that are subsequently digested with calcium bisulphite, using heat and pressure. The sulphite liquor reaches a temperature of 130 to 140°C. toward the end of the process. In some processes the temperature may rise even higher, with the result that some of the sugar in the liquor is destroyed. The cellulose pulp obtained by this method is used for the manufacture of paper.

[1] McGlumphy, J. H., J. W. Eichinger, R. M. Hixon, and J. H. Buchanan, *Ind. Eng. Chem.*, **23**: 1202 (1931).

[2] Underkofler, L. A., W. K. McPherson, and E. I. Fulmer, *Ind. Eng. Chem.*, **29**: 1160 (1937).

b. Composition of Sulphite Liquor.—Together with each ton of pulp, there is produced 8 to 10 tons of sulphite liquor, which contains from 10 to 12 per cent of total solids. One analysis[1] of sulphite liquor showed the presence of the following products for each 1,000 kg. of cellulose obtained from Swedish spruce: lignin, 644 kg.; carbohydrates, 311 kg.; proteins, 15 kg.; resin and fats, 73 kg.; sulphur dioxide combined with lignin, 235 kg.; and calcium oxide combined with lignosulphonic acid, 102 kg.

The carbohydrates consisted of 49.4 per cent glucose, 15.6 per cent mannose, 8.1 per cent galactose, and 26.9 per cent nonfermentable pentosans (arabinose).

Approximately 65 per cent of the total reducing sugars are usually fermentable.

c. Treatment of Sulphite Liquor.—Before the liquor is inoculated with yeast it is necessary to remove or neutralize the sulphur dioxide, acetic acid, and formic acid that are present. The methods commonly employed to accomplish this are (1) steam-stripping followed by neutralization with lime or calcium carbonate; or (2) direct neutralization.

d. Production.—The production of ethyl alcohol from sulphite waste liquor has been described by Foth, Sankey, and Rosten (1944), Joseph (1947), Eriesson (1947), and others.

Ethyl alcohol is produced commercially from sulphite liquor in this country, Canada, Germany, Sweden, and other countries.

e. Process at Bellingham, Wash.—Eriesson[2] described the production of ethyl alcohol from sulphite waste liquor at the Bellingham plant. Figure 30 shows a flow diagram for the process.

COLLECTION OF LIQUOR.—Sulphite waste liquor from the digesters is discharged, together with wood pulp fibers, into blow pits that have perforated bottoms or plates of stainless steel. The sulphite liquor is drained off and stored in a tank at a temperature of about 90°C.

CONDITIONING.—From the storage tank the sulphite liquor is pumped to the top of a column and flows downward over stainless-steel perforated plates, while steam introduced at the bottom of the column and flowing upwards removes sulphur dioxide and other volatile materials. The sulphur dioxide saved is reused. The hot sulphite liquor is pumped through screens which remove wood pulp fibers. It flows by gravity to a storage tank. Passage of the liquor through flash coolers reduces its temperature to 30°C. and concentrates it by about 10 to 12 per cent. A slurry of lime is added to adjust the pH to 4.5. Urea is added to the liquor as a source of nitrogen.

[1] EWESON, E. W., *Chem. Inds.*, **38**: 573 (1936).

[2] ERIESSON, E. O., *Chemical Eng. Progress, Trans. Sect.*, **43** (No. 4): 165 (1947).

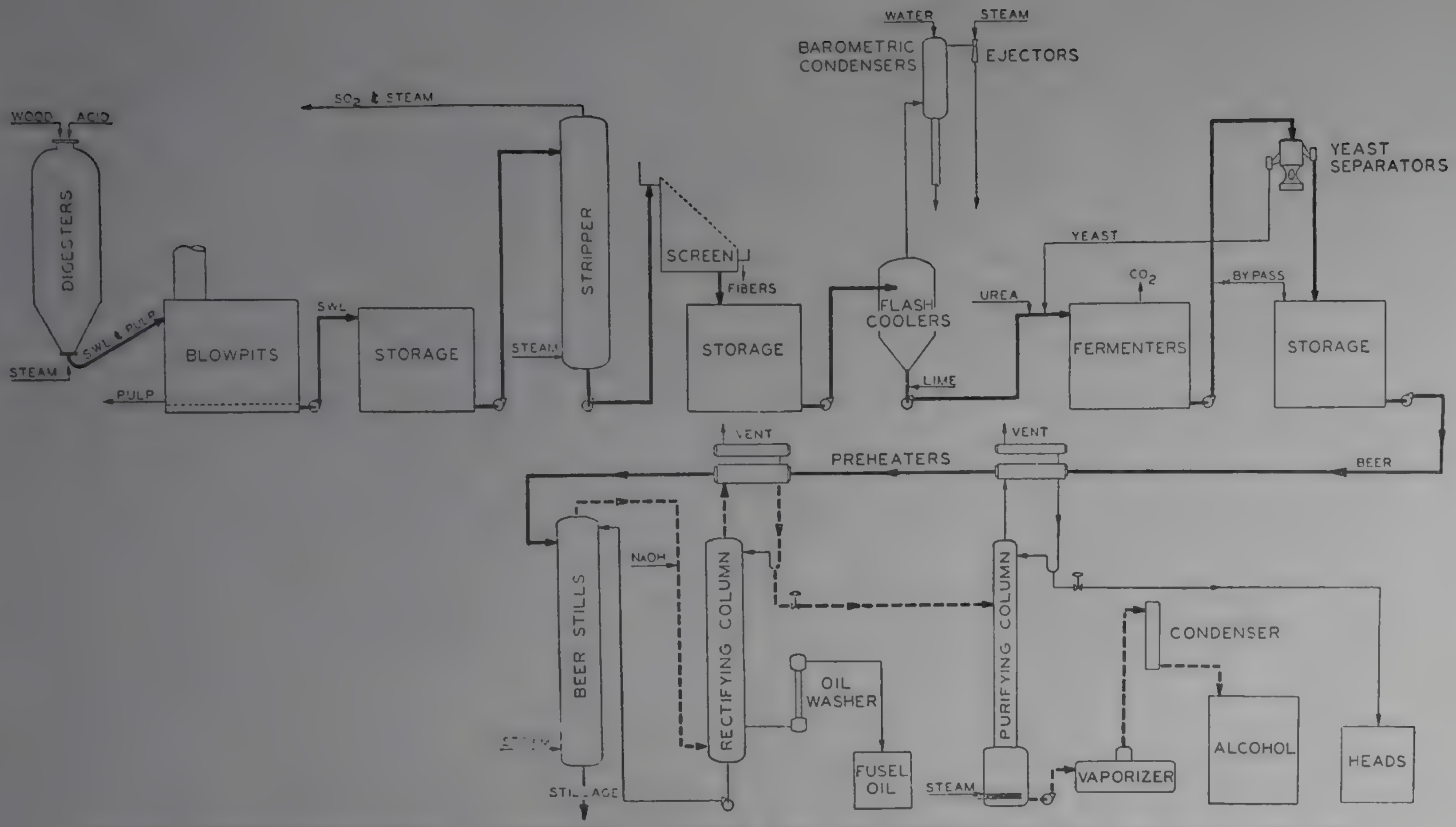


FIG. 30.—Generalized flow diagram. [*Courtesy of E. O. Ericsson, Chem. Eng. Progress (Trans. Sect.)*, **43** (No. 4): 165 (1947).]

FERMENTATION.—The conditioned sulphite liquor is pumped into the first of a series of seven interconnected fermentation tanks. Yeast from a previous cycle is mixed with the sulphite liquor as it flows to the fermenter. The medium is agitated in each tank and the flow of liquor is on a continuous basis. Fermentation is carried out at 30°C.

SEPARATION OF YEAST.—After the fermentation is over, in about 20 hr., the fermented liquor is pumped to yeast separators. The required amount of yeast is returned to the first fermenter for reuse.

FINAL OPERATIONS.—The clarified liquor (beer) is passed by gravity to a storage tank. Then it is distilled. Finally the alcohol is rectified to 190 proof or higher, purified, and warehoused.

YIELD.—The yield is 22 gal. of alcohol per ton of pulp.

CAPACITY OF PLANT.—The capacity of the plant is 6,500 gal. (or more) of alcohol per day.

f. The Melle Process.—This process was developed by Les Usines de Melle[1] and involves the "reuse of yeast." The yeast from a completed alcohol fermentation is separated from the alcohol and spent liquor by means of centrifugal separators. This yeast is used again and again with usually no appreciable loss of fermentation ability. The reuse of yeast is particularly important in the alcoholic fermentation of sulphite liquor, where large quantities of yeast are essential for rapid and efficient fermentations.

8. Ethyl Alcohol from Wood Sugars.—In order to obtain maximum yields from wood sugars, it is essential to remove inhibiting substances and to add necessary nutrients.

Several actions may be taken to remove inhibiting materials. For example, Leonard and Hajny[2] found that the toxic character of the wood-sugar solutions could be counteracted by the addition of reducing agents, such as Na_2SO_3, $NaHSO_3$, $Na_2S_2O_3 \cdot 5H_2O$, $Na_2S_2O_5$, $Na_2S_2O_4$, $KHSO_3$, Na_2S, sulphite waste liquor, alkali decomposed sugar, ascorbic acid, cysteine, and reduced-iron filings. The same effect could be secured by heating neutral solutions. The amount of dissolved calcium sulphite was decreased by neutralization at about 140°C. For good alcohol yields and avoidance of sugar losses, heating should be for 15 to 30 min. at 140°C. with the solution at a pH of 4.5 to 5.2. Toxic substances were removed from the wood hydrolyzate by steam distillation, which was particularly beneficial when a quantity of greater than 0.1 per cent of furfural was present.

Leonard and Hajny[2] found the following method of treating and

[1] Canadian Patents 341,720; 348,549; 402,847.

[2] LEONARD, R. H., and G. J. HAJNY, *Mimeo, No. R* 1466, *U.S. Dept. Agr., Forest Products Lab.*, December, 1944.

fermenting wood sugars to be successful: The acid hydrolyzate was neutralized at room temperature to a pH of 5.0 ± 0.2 with a slurry of calcium carbonate. The product was filtered and 0.03 per cent by weight of sulphur dioxide as Na_2SO_3, $Na_2S_2O_5$, or similar reducing agent, was added. The mixture was heated for 15 min. at 135 to 140°C. in a bomb, drawn, and cooled to 30°C. The pH of the medium was adjusted to 5.8 with sodium hydroxide. Nutrients, 0.02 per cent urea and 0.006 per cent NaH_2PO_4 by weight, were added and the medium was inoculated with 2 per cent by volume of fresh yeast (*S. cerevisiae* No. 49 of the University of Wisconsin collection) and agitated. The fermentation was usually complete in less than 20 hr. when the initial sugar concentration, as glucose, was 5 to 7 g. per 100 ml.

Harris and associates[1] studied the fermentation of Douglas fir hydrolyzates. They observed that the fermentation time was 5 to 6 hr. when the inoculum was 2 per cent by dry weight of *S. cerevisiae* (No. 49 University of Wisconsin) and 24 hr. when the inoculum was 0.5 per cent by dry weight. The yeast from an 18- to 20-hr. fermentation was used to inoculate a fresh lot of medium containing 5 per cent sugar. At the end of this fermentation the yeast was separated out and used to inoculate a new batch. This sequence was followed 59 times without loss in alcohol yield and without contamination with bacteria and other yeasts or molds. It was possible to increase the rate of fermentation but not the yield of alcohol from wood sugars by the addition of 0.025 per cent Louisiana second-crop molasses. They obtained yields of 39.2 to 40 per cent based on total sugar and 47.0 to 47.9 per cent based on fermented sugar. In terms of a 5 per cent sugar solution, a yield of 2.64 gal. of 95 per cent alcohol per 100 gal. of hydrolyzate was obtained. From 1 ton of dry bark-free wood, 64.5 gal. of alcohol were produced.

Two-bushel-per-Day Continuous Alcohol Unit.—Altsheler and his associates[2] have designed a continuous-process unit capable of producing 5 gal. of 190-proof alcohol in 24 hr. from 2 bu. of grain. A flow diagram of the process employed is shown in Fig. 31. The process is as follows:

Corn is transported from a bin by a screw-conveyor to a Raymond hammer mill where it is ground in such a manner that substantially all of it passes through a 20-mesh screen. The meal is blown by the mill to a cyclone separator from which it falls to a slurry vessel made of Pyrex glass. It is mixed with enough water to provide a ratio of about 23 gal.

[1] Harris, E. E., G. J. Hajny, M. Hannan, and S. Rogers, *Mimeo. No. R* 1618, *U.S. Dept. of Agriculture, Forest Products Lab.*, June, 1946.

[2] Altsheler, W. B., H. W. Mollet, E. H. C. Brown, W. H. Stark, and L. A. Smith, *Chem. Eng. Progress, Trans. Sect.*, **43** (No. 9): 467 (1947).

per bu. Sufficient sulphuric acid is added to produce a weight concentration of 1.25 per cent.

The mash is forced to a cooker tube, which is 1.5 in. in diameter and made of copper, by a piston pump. Steam at a pressure of 150 lb. per sq. in. heats it almost instantly to 340°F. It is held in the cooker for 6 min., and then neutralized by a slurry of calcium carbonate. It is then cooled to 88 to 90°F. in a heat exchanger (20 ft. of 0.25-in. copper tubing in the form of a helix with cooling water on the outside), and discharged to the first of two fermenters.

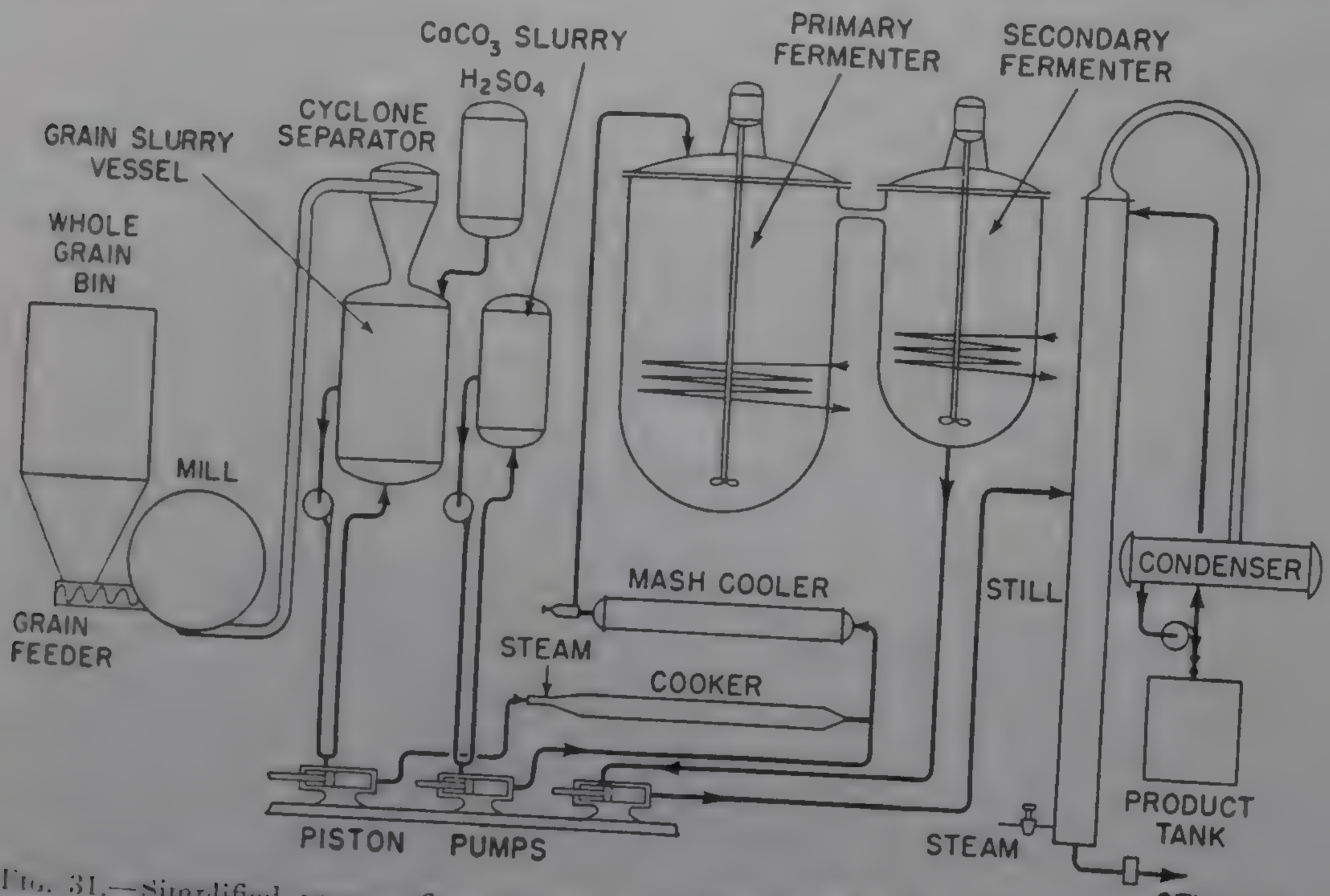


FIG. 31.—Simplified process flow diagram. [*Courtesy of W. B. Allsheler, H. W. Mollet, E. H. C. Brown, W. H. Stark, and L. A. Smith, Chem. Eng. Progress (Trans. Sect.)*, **43** (No. 9): 467 (1947).]

Fermentation is continuous and is carried out in Pyrex-glass vessels each of which is equipped with a stirrer. The primary fermenter is equipped with cooling coils and has a working capacity of 27 gal. It is connected at the top with the secondary fermentor by means of an overflow pipe. The mash remains about 8 hr. in the primary fermentor and about 3 hr. in the secondary fermentor.

The concentration of the mash as it enters the primary fermentation tank is 1 bu. of corn in 40 gal. Yeast is added to the mash when the process is first started, but the rate of reproduction is sufficiently rapid thereafter to obviate the necessity of further additions. The usual concentration of cells is 100,000,000 per milliliter. The temperature is

TABLE 29.—AVERAGE ANNUAL PRODUCTION OF CERTAIN AGRICULTURAL BY-PRODUCTS FOR 1931–1935[1]

By-product	Grain per bushel, pounds	Dry by-product per pound of grain, pounds	Dry by-product per bushel of grain, tons	Average production of dry by-product, 1,000 tons	Estimated quantity of dry by-product available for industrial use, 1,000 tons
Wheat straw	60	1.9	0.057	38,794	29,000
Rye straw	56	2.5	0.070	2,378	1,800
Oat straw	32	1.3	0.0208	20,156	0
Barley straw	48	1.2	0.0283	5,965	4,474
Flax straw	56	4.0	0.112	1,128	1,128
Rice straw	45	1.2	0.027	1,089	820
Total straws	..	....	.....	69,510	37,222
Corncobs	56	0.22	0.00616	12,408	1,400
Oat hulls	32	0.30	0.0048	4,651	150
Rice hulls	45	0.20	0.0045	182	182
Cottonseed hulls	..	....	.....	1,165*	583
	..	....	.....	183†	92
Total cobs and hulls	..	....	.....	18,589	2,407
Corn stover	56	1.2	0.0336	63,681	28,500
Cotton stems and pods	..	....	.....	17,544‡	12,281
Bagasse fiber, continental United States	..	....	.....	423§	423
Bagasse fiber, insular United States	..	....	.....	3,276‖	3,276
Total other by-products	..	....	.....	84,924	44,480
Grand total	..	....	.....	173,023	84,109

[1] Senate Document 65, 76th Congress, 1st Session, "A Report of a Survey Made by the Department of Agriculture Relative to Four Regional Research Laboratories, One in Each Major Farm Producing Area," Washington, D.C., 1939.

* Reported production, but not limited to crop years specified.
† One-third of in-the-hull production.
‡ Calculated; 2:1 used as ratio of stems and pods to combined seed and lint.
§ Cane production ×0.125 for Louisiana and Florida.
‖ Cane production ×0.126 for Hawaii; ×0.12 for Puerto Rico and Philippine Islands.

maintained at 88 to 90°F. in the primary fermentor by means of water circulating through coils. The pH is 4.4 to 4.6.

Alcohol is stripped from the beer by 20 perforated plates located in the lower section of the still and rectified by 26 bubble-cap plates in the upper section of the still. The alcohol vapors from the top of the column travel to a condenser, which is cooled by water.

For further details, the reader is referred to the original publication by Mehlitz and his coworkers.[1]

Cost of Producing Ethyl Alcohol.—The cost of producing ethyl alcohol from various raw materials varies considerably and is determined more or less by world conditions. Tousley[2] has discussed this subject in some detail.

Cellulosic Wastes.—Each year millions of tons of cellulose-containing materials are permitted to go to waste in the United States. In Table 29 are presented estimates concerning the production of various by-products which may be termed agricultural wastes or residues. These data are subject to variation from year to year, since crop production is not stable.

TABLE 29.—AVERAGE YIELDS OF 95.5 PER CENT ALCOHOL PER TON[2]

Material	Gallons	Material	Gallons
Wood (all species)	87.0	Yams	27.3
Oats	84.0	Potatoes	22.9
Barley straw	83.4	Sugar beets	22.1
Straw	80.4	Figs, fresh	21.0
Hay, mixed grasses	79.5	Jerusalem artichokes	20.0
Oat straw	79.5	Pineapples	15.6
Barley	79.2	Sugar cane	15.2
Hay, dry	79.0	Grapes (all varieties)	15.1
Rye	78.8	Apples	14.4
Prairie hay	72.0	Apricots	13.6
Wheat, Hard Red	70.4	Pears	11.5
Sorghum stover	70.4	Peaches	11.5
Corn	69.6	Fresh tomatoes	10.9
Flax straw	70.0	Carrots	9.8
Sweet potatoes	64.2		

[1] Jacobs, P. B., and H. P. Nees, U.S. Dept. of Agr. Misc. Pub. 457, December, 1941.

Yields from Various Raw Materials.—The probable average yield of 95.5 per cent ethyl alcohol per ton of raw material is shown in Table 30. Table 31 supplies information concerning the probable average yield of ethyl alcohol per acre from various farm crops.

Government Supervision.—The purpose of Government supervision is to prevent the illegal use of untaxed alcohol. Ethyl alcohol is taxed, as noted, to provide revenue for the Government. Should tax-free ethyl alcohol be used for beverage purposes, the tax from such alcohol would be

[2] Tousley, R. D., *Chem. Met. Eng.*, **52** (No. 10): 120 (October, 1945

Government supervision is carried out by Government officers who are stationed at all the manufac uring plants, the bonded warehouses, and the denaturing plants, none of which can be operated without permit from the Treasury Department. The officers keep records of the raw materials used, of the yields of ethyl alcohol, of the transfers of alcohol to the bonded warehouse, of transfers of alcohol from the bonded warehouse to the denaturing plant, and of the final disposal of the

TABLE 31.—AVERAGE YIELD[1] OF 99.5 PER CENT ALCOHOL PER ACRE[2]

Material	Gallons	Material	Gallons
Sugar cane (Hawaii, 18 to 22 months)	889.0	Pineapples	78.0
Sugar beets	287.0	Rice, rough	65.6
Sugar cane (Louisiana)	268.0	Pears	49.3
Jerusalem artichokes	180.0	Barley	47.9
Potatoes	178.0	Molasses, blackstrap	45.0
Sweet potatoes	141.0	Apricots	41.0
Apples	140.0	Oats	36.3
Dates, dry	126.0	Grain sorghum	35.5
Carrots	121.0	Buckwheat	34.2
Raisins	101.7	Wheat (all varieties)	33.0
Yams	94.0	Figs, fresh	31.5
Grapes (all varieties)	90.4	Figs, dry	29.5
Corn	88.8	Sorghum cane	26.4
Peaches	84.0	Rye	23.8
Prunes, dry	82.8	Plums (nonprunes)	21.8

[1] Probable yield calculated from the average fermentable content and based on present over-all average acre yield in the United States.

[2] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

alcohol. The keys to the bonded warehouse are held by the Government officers. (For further details, consult Regulations 3, U.S. Department of the Treasury.)

Tax-paid Alcohol.—Tax-paid pure ethyl alcohol may be purchased without permit or bond for manufacturing purposes. State regulations must be complied with, however.

Tax-free Alcohol.—Alcohol " . . . may be withdrawn under regulations from any industrial plant or bonded warehouse tax free by the United States or any governmental agency thereof, or by the several States and Territories, or any municipal subdivision thereof, or by the District of Columbia, or for the use of any scientific university or college of learning, any laboratory for use exclusively in scientific research, or for use in any hospital or sanitarium."[1]

[1] U.S. Dept. of the Treasury, Bureau of Industrial Alcohol, Regulations 3, 1931.

A person entitled to obtain tax-free alcohol, governmental agencies excepted, must first obtain a permit to purchase and then furnish bond as prescribed in Sections 3100–3124 of the Internal Revenue Code.

Denatured Alcohol.—Alcohol for use in many industries is denatured by bonded denaturing plants. "A denatured alcohol is ethyl alcohol to which has been added such denaturing materials as render the alcohol unfit for use as an intoxicating beverage."[1] Denatured alcohol may be completely denatured or specially denatured. A completely denatured alcohol is one that has been rendered totally unfit for beverage purposes by the addition of the substances specified in one of the formulas provided by the Bureau of Internal Revenue. Completely denatured alcohols may be used as antifreeze agents, for alcohol lamps, in insect powders, and for many other purposes. Such alcohol is available to manufacturers or the general public without obtaining a permit or filing a bond and, if sold and used according to the law and regulations. is not subject to the internal revenue tax.

Specially denatured alcohol is ethyl alcohol that has been rendered unfit for use as a beverage by the addition of one or more substances, but which is adapted for use in a large number of specialized industries or arts. Methyl alcohol (CH_3OH), benzol, pine tar (Pix liquida, U.S.P.), vinegar (containing not less than 9 per cent acetic acid), and ethyl ether are some of the denaturants which may be added to ethyl alcohol to render it specially denatured.[2] Specially denatured alcohol is "sold, possessed and used only pursuant to permit and bond, except as otherwise provided."[2]

According to the U.S. Treasury Department, there were 36,395,715 wine gallons of completely denatured alcohol and 147,348,371 wine gallons of specially denatured alcohol produced by the United States (including Hawaii and Puerto Rico) during the fiscal year 1947.

Table 32 shows some important uses of industrial alcohol.

Fusel Oil.—Fusel oil, which forms 0.1 to 0.7 per cent of the crude distilled spirit, is a mixture of amyl and isoamyl alcohols, principally; with smaller quantities of isobutyl and normal propyl alcohols and traces of acids, esters, and aldehydes. The following typical analysis[3] is given by Delbrück for the fusel oil obtained from the fermentation of potatoes: amyl alcohol, 68.76 per cent; isobutyl alcohol, 24.35 per cent; *n*-propyl alcohol, 6.85 per cent; and acids, esters, and furfural, 0.04 per cent. The

[1] U.S. Dept. of the Treasury, Bureau of Internal Revenue, Appendix to Regulations 3, 1939.

[2] *Ibid.*

[3] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

composition and the quantity of fusel oil formed varies, however, according to the raw materials used and the nature of the fermentation. For example, 1 gal. of fusel oil may be obtained for each 1000 gal. of ethanol in the fermentation of molasses,[1] but larger quantities may be obtained from potatoes and corn.

Fusel oil is used principally as a lacquer solvent. It is usually not refined or separated into its components.

A further discussion of some of the constituents of fusel oil will be found in the following chapter.

References on the Production of Industrial Alcohol and Related Subjects

ALTSHELER, W. B., H. W. MOLLET, L. H. C. BROWN, W. H. STARK, and L. A. SCHAD: Design of a Two-bushel per Day Continuous Alcohol Unit, *Chem. Eng. Progress Trans. Sect.*, **43** (No. 9): 467–472 (1947).

ANON.: Alcohol on the Hoof, Seagram's Designs Mobile Continuous Process Alcohol Unit, *Chem. Inds.*, October, 1946.

ANON: Former Waste Product Now Yields 7,500 Gallons of Alcohol Daily, *Int. Assoc. Centrifugal Rev.*, **13** (No. 2): 3–4 (1947).

ANON: General Plans of Operation and List of Equipment for Whey Fermentation by Yeast, BDIM-1011, *Bureau of Dairy Industry*, U.S. *Dept. Agr.*, May, 1947.

ATWATER, W. O., and A. P. BRYANT: The Chemical Composition of American Food Materials, *U.S. Dept. Agr., Bull.* 28, rev. ed., 1906.

BARROS, H.: "Distillation of Alcohol," *Division of Education*, Joseph E. Seagram & Sons, Inc., Louisville, Ky., 1944.

BERNHAUER, K.: "Gärungschemisches Praktikum," 2d ed., Verlag Julius Springer, Berlin, 1939.

BILFORD, H. B., R. E. SCALL, W. H. STARK, and P. J. KOLACHOV: Alcoholic Fermentation of Molasses, Rapid Continuous Fermentation Process, *Ind. Eng. Chem.*, **34**: 1406–1410 (1942).

BORUFF, C. S., and J. M. VAN LANEN: The Fermentation Industry during World War II, *Ind. Eng. Chem.*, **39**: 934–937 (1947).

BOSWELL, V. R., C. E. STEINBAUER, M. F. BABB, W. L. BURLISON, W. H. ALLEMAN, and H. A. SCHOTH: Studies on the Culture and Certain Varieties of the Jerusalem Artichoke, *U.S. Dept. Agr., Tech. Bull.* 514, May, 1936.

BROWNE, H. H.: Ethyl Alcohol from Fermentation of Lactose in Whey, *Ind. Eng. Chem., News Ed.*, **19**: 1271 (1941).

CHATFIELD, C., and G. ADAMS: Proximate Composition of Fresh Vegetables, *U.S. Dept. Agr., Circ.* 146, 1931.

——— and I. I. MCLAUGHLIN: Proximate Composition of Fresh Fruits, *U.S. Dept. Agr., Circ.* 50, 1928.

CLAASSEN, H.: Die Wirtschaftlichkeit der Herstellung von Holzzucker, *Chem. Ztg.*, **56**: 989 (1932).

COLEMAN, D. A. (chairman): "Methods for the Analysis of Cereals and Cereal Products. Reference Tables," American Association of Cereal Chemists, Lancaster, Pa., 1928.

DE BECZE, G., and M. ROSENBLATT: Continuous Fermentation, *Am. Brewer*, **76** (No. 2): 11 (1943).

[1] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

Scholler, H.: Die Gewinnung von Zucker, Spiritus und Futterhefe aus Holz als Rohstoff, *Chem. Ztg.*, **60**: 293 (1936).

Sherwood, J. R.: Some Industrial Fermentations, *Australian Chem. Inst. Jour. & Proc.*, **14**: 221–232 (June, 1947).

Skinner, W. W.: Industrial Alcohol, *Report No. AIC*-3, *NM*-235, *U.S. Dept. Agr.*, March, 1943.

Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov: Laboratory Cooking, Mashing, and Fermentation Procedures, *Ind. Eng. Chem., Anal. Ed.*, **15**: 443–446 (1943).

———, P. Kolachov, and H. F. Willkie: Wheat as a Raw Material for Alcohol Production, *Ind. Eng. Chem.*, **35**: 133–137 (1943).

Thorne, C. B., R. L. Emerson, W. J. Olson, and W. H. Peterson: Evaluation of Malts for Production of Alcohol from Wheat, *Ind. Eng. Chem.*, **37**: 1142–1144 (1945).

Tousley, R. D.: Alcohol Economics Will Determine Future Production Processes, *Chem. Met. Eng.*, **52** (No. 10): 120–123 (October, 1945).

Underkofler, L. A., W. K. McPherson, and E. I. Fulmer: Alcoholic Fermentation of Jerusalem Artichokes, *Ind. Eng. Chem.*, **29**: 1160 (1937).

U.S. Dept. of Agriculture, Northern Regional Research Laboratory: The Production of Ethyl Alcohol from Wheat, March, 1944.

U.S. Dept. of the Treasury, Bureau of Industrial Alcohol, Regulations 3. Relative to the Production, Tax Payment, etc., of Industrial Alcohol and to the Manufacture, Sale, and Use of Denatured Alcohol, 1931.

U.S. Dept. of the Treasury, Bureau of Internal Revenue, Formulae for Completely and Specially Denatured Alcohol. Appendix to Regulations 3, 1939.

Willkie, H. F., and P. J. Kolachov: "Food for Thought," Indiana Farm Breau, Inc., Indianapolis, 1942.

Some Patents

Bergius, F., H. Koch, and H. Zimmermann: Process for Obtaining Alcohol by Fermentation of Liquids Containing Carbohydrate, U.S. Patent 2,146,326, Feb. 7, 1939.

Christensen, L. M., and E. I. Fulmer: Production of Ethyl Alcohol from Artichoke Tubers, U.S. Patent, 2,085,003, 1937.

Hildebrandt, F. M.: Alcoholic Fermentation Process and Product Thereof, U.S. Patent 1,921,991, Aug. 8, 1933.

Karsch, W.: Fermentation Process, U.S. Patent 2,155,134, Apr. 18, 1939.

Kitzmeyer, E. L.: Method of Propagation of Culture Yeast, U.S. Patent 2,150,329, Mar. 14, 1939.

Legg, D. A.: Yeast Fermentation Process, U.S. Patent 2,026,237, Dec. 31, 1935.

Magné, J. H. P.: Apparatus for Cultivating Yeast, Mucorini, Molds, or Bacilli, U.S. Patent 1,212,656, 1917.

Plaček, A.: Process for Producing Ethyl Alcohol from Wood or Other Cellulose-containing Materials, U.S. Patent 1,888,935, Nov. 22, 1932.

Scholler, H.: Purifying Wood-sugar Wort, German Patent 676967, June 16, 1939.

Schwarz, R., and A. R. Erda: Pure Culture Apparatus, U.S. Patent 2,147,271, Feb. 14, 1939.

Wagner, T. B.: Manufacture of Ethyl Alcohol from Indian Corn, U.S. Patent 2,132,250, Oct. 4, 1938.

Wickerham, L. J.: A Method for Producing Ethyl Alcohol, U.S. Patent 2,431,004, Nov. 18, 1947.

CHAPTER V

FACTS AND THEORIES CONCERNING THE MECHANISM OF ETHYL ALCOHOL FERMENTATION

Some Early Researches.—In the earliest studies involving the mechanism of the ethyl alcohol fermentation, one was concerned only with the initial and end products. Black,[1] in the eighteenth century, stated that ethyl alcohol and carbon dioxide were the only products formed from sugar during the ethanol fermentation. Lavoisier, in 1789, carried out quantitative studies on this fermentation and found, besides ethyl alcohol and carbon dioxide, another product to which he gave the name "acetic acid." From 95.9 parts of sugar he obtained 57.7 per cent ethyl alcohol, 33.3 per cent CO_2 and 2.5 per cent acetic acid. Lavoisier's is believed to be the first quantitative work concerning the ethanol fermentation, although his results were incorrect.

Gay-Lussac investigated more intensively the field of research opened up by Lavoisier. He formulated the following equation to show what he believed was the essential chemical reaction of the ethanol fermentation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} = 2\underset{\substack{\text{Ethyl}\\\text{alcohol}}}{C_2H_5OH} + 2\underset{\substack{\text{Carbon}\\\text{dioxide}}}{CO_2}$$

From 45 parts of glucose, he obtained 23 parts of alcohol and 22 parts of carbon dioxide.

By 1857, Pasteur had begun to publish the results of his researches on fermentation. He showed that 100 parts of sucrose gave rise to 105.4 parts of invert sugar, which in turn yielded 51.1 parts of ethyl alcohol, 49.4 parts of carbon dioxide, 3.2 parts of glycerol, 0.7 part of succinic acid, and 1 part of other substances. He thus proved that the weight relationships set forth by Gay-Lussac were in error.

Buchner,[2] in 1897, prepared a cell-free juice from yeast (by grinding the cells with sand and subjecting them to high pressure) which possessed the ability to produce ethyl alcohol and carbon dioxide from sugar. This discovery lent great impetus to subsequent studies concerning the intermediate reactions in the formation of ethyl alcohol.

[1] FREY, C. N., *Ind. Eng. Chem.*, **22:** 1154 (1930).
[2] *Ber.*, **30:** 117, 1110 (1897).

Hexose Phosphates.—Harden and Young[1] (1905) discovered the importance of phosphates in cell-free fermentation. They have demonstrated that inorganic phosphates disappear during the first part of the fermentation, while organic phosphates, *i.e.*, esters of hexose, are produced. They have described hexosediphosphate and proposed the following fundamental equation for fermentation by yeast extract:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2C_2H_5OH + 2CO_2 + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$$

Hexosediphosphate is an important intermediate in the schemes of Embden, Meyerhof, and others for the breakdown of carbohydrates. This compound contains two phosphate, or phosphoric acid, molecules attached, one each, to the first and sixth carbon atoms of the hexose, which appears to be fructose. The same hexosediphosphate is obtained from glucose, fructose, or mannose.

At least two hexosemonophosphates, which have different properties, have been prepared: the Robison ester (glucopyranose-6-phosphoric ester), and the Neuberg ester (fructofuranose-6-phosphoric ester). When these esters are hydrolyzed, they yield glucose and fructose, respectively.[2]

Hexosemonophosphates have been synthesized by Levene and Raymond, Smythe, and others. A trehalosemonophosphate has been isolated by Robison and Morgan from a fermentation brought about by dried yeast.

Hexose phosphates are fermented at different rates. The diphosphate is fermented more slowly than glucose, while hexosemonophosphate is usually fermented more rapidly than the diphosphate, at a rate comparable with glucose in the initial stage only.[3]

The addition of hexose phosphates to cell-free yeast extracts containing glucose removes or greatly shortens the period of induction (the pause before fermentation starts). Without phosphates, no fermentation takes place.

The addition of phosphates to a medium does not affect the rate of fermentation by living yeasts.

For further details in respect to this very important subject, the reader is referred to Harden's monograph, "Alcoholic Fermentation," and to the bibliography that follows this chapter.

Methods of Studying the Mechanism of Fermentation.—Information concerning the mechanism of the ethyl alcohol fermentation may be

[1] *Jour. Physiol.*, **32** (Proc., Nov. 12, 1904), 1905.

[2] GORTNER, R. A., "Outlines of Biochemistry," 2d ed., John Wiley & Sons, Inc., New York, 1938.

[3] MICHAELIS, L., *Ind. Eng. Chem.*, **27**: 1037 (1935).

gained by studying the related mechansim of lactic acid formation by muscle extracts; by the use of cell-free yeast juice or extracts, fixation methods, selective poisons, and dialysis; and by other means.

1. *Related Mechanism of Lactic Acid Formation by Muscle Extracts.*—Any productive advance in the study of the mechanism of lactic acid formation by muscle extract has usually aided materially in the study of the mechanism of the ethyl alcohol fermentation, and vice versa. The researches of Embden on muscle extract exemplify the impetus that research on the former may do for the latter. The outstanding work of Meyerhof and his school add further proof to the valuable insight gained through correlative studies.

2. *Cell-free Extracts.*—Since the important reactions that take place in the conversion of sugar to ethyl alcohol occur normally within the living cell, since added hexose phosphates are fermented extremely slowly or not at all, and since the isolation of intermediates from the living cell is impossible in many cases, but comparatively little information concerning the intermediary reactions may be derived from studying such cells. Accordingly, cell-free extracts are used.

Two common methods of preparing the extract are the Buchner method (already mentioned) and the Lebedev[1] method. In the latter method the yeast is washed thoroughly with water, dried at 25 to 30°C., rubbed through a sieve, dried further, and stored until such time as an extract may be required. The extract is prepared by mixing 1 part by weight of dried yeast with 3 parts of water, incubating at 37°C. for 3 hr. and filtering the extract into a vessel cooled by ice.[2] It is important to use an extract that is free from living cells.

3. *Fixation Method.*—The fixation method was used with success by Neuberg. In this method a sulphite, such as calcium sulphite, or dimedon (dimethyl cyclohexane-dione), is used to fix the acetaldehyde as it is formed. The fixation product is not fermented by yeast and therefore accumulates in the medium. Since acetaldehyde is a hydrogen acceptor, the removal of this substance from the fermentation medium causes other hydrogen acceptors to become active. Use is made of this fact in the production of glycerol by fermentation, where the role of sulphites is very important.

Although pyruvic aldehyde forms an addition product with sulphites, this product is fermentable.

4. *Selective Poisons.*—Certain reactions in the normal chain of enzyme reactions do not take place in the presence of selective poisons, such as

[1] LEBEDEV, A. VON., *Compt. rend.*, **152:** 49, 1129 (1911).

[2] STEPHENSON, M., "Bacterial Metabolism," 2d ed., Longmans, Green & Company, New York, 1939.

monoiodoacetic acid, sodium fluoride, and other substances. The poisons affect specific enzyme systems.

When monoiodoacetic acid is added to a cell-free extract containing hexosediphosphate, a mixture of triose phosphates forms as usual, which contains dihydroxyacetone phosphate and glyceraldehyde phosphate, but the reaction, in which triose phosphate is oxidized to phosphoglyceric acid with the simultaneous reduction of acetaldehyde to ethyl alcohol, does not take place, owing to a poisoning of the enzyme system. Monoiodoacetic acid does not, however, retard the decomposition of phosphoglyceric acid.

Sodium fluoride, on the other hand, prevents phosphoglyceric acid from being broken down to pyruvic and phosphoric acids, but it does not prevent triose phosphate from being converted to phosphoglyceric and glycerophosphoric acids. Accordingly, phosphoglyceric acid accumulates in the medium.

5. *Dialysis.*—By means of dialysis, important constituents of yeast extract may be separated and studied.

The adenylic acid system and magnesium may be removed from yeast extract by dialysis. It is thus possible to study the reactions in which a gain or loss of phosphates is concerned.

Proteins, separated from coenzyme systems by dialysis, may be fractionated in the ways common to biochemistry.

6. *Other Means.*—There are other ways in which the intermediary reactions of fermentation may be studied.

Kluyver, Van der Lek, and others have demonstrated the value of studying the quantitative relationships of the intermediate and end products in formulating a scheme for the mechanism of fermentation.

Lohmann has obtained helpful information by determining the time required for normal hydrochloric acid to split off the phosphates from phosphorylated compounds.

Important information has been secured by studying the fermentability and fermentation rates of substances assumed to be intermediates.

A study of the formation and utilization of hexosemonophosphate has been made possible through the use of selected dyes. Naphthosulphonate indophenol, brilliant alizarin blue or rosindulin GG inhibit the fermentation of glucose or of hexosemonophosphate but not of hexosediphosphate by yeast extract under aerobic conditions.[1] When hexosediphosphate is added to yeast extract containing glucose and one of these dyes, the hexosediphosphate is fermented. Contrary to expectations. the quantity of inorganic phosphate does not increase with the fermenta-

[1] MICHAELIS, L., and C. V. SMYTHE, *Jour. Biol. Chem.*, **113: 717** (1936).

tion of hexosediphosphate, but it actually decreases, for an organic phosphate is synthesized. The reaction may be expressed as follows:

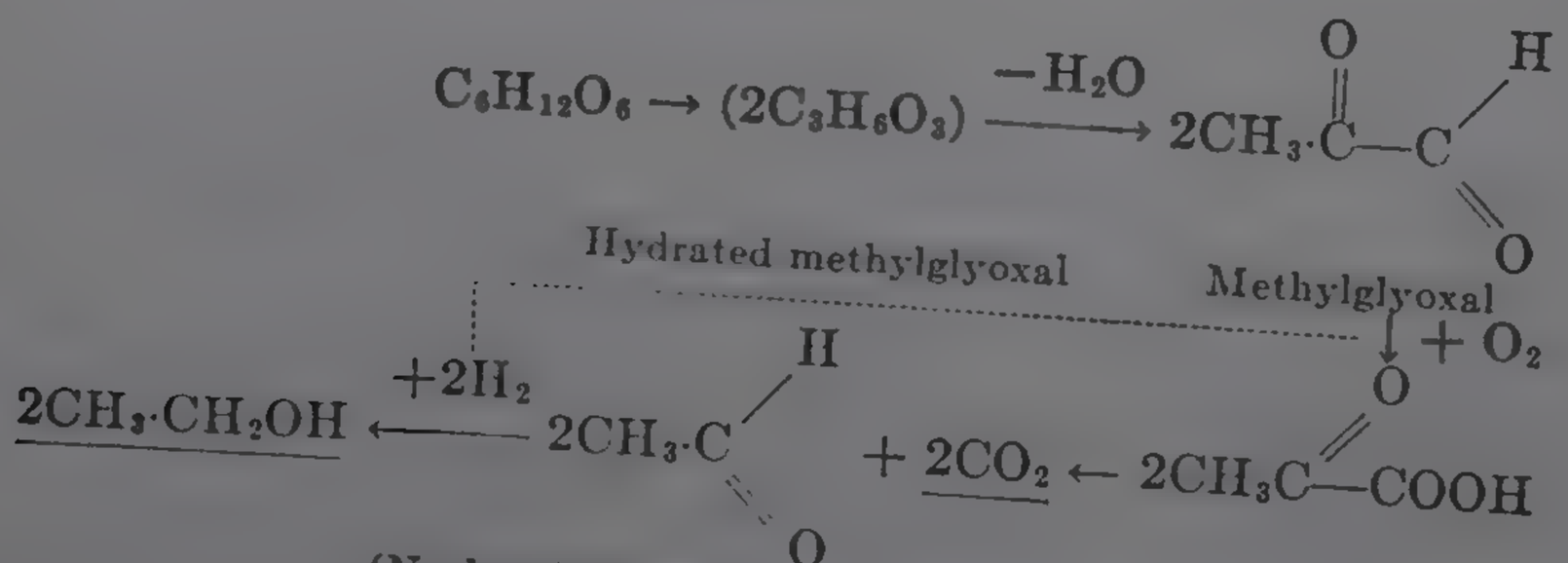
$$C_6H_{10}O_4(PO_4M_2)_2 + 2M_2HPO_4 + 4ROH \rightarrow 4RPO_4M_2 + 2CH_3CH_2OH + 2CO_2 + 2H_2O^*$$

The organic phosphate (RPO_4M_2) formed in the preceding equation appears to be hexosemonophosphate, a mixture of glucose, fructose, and mannose monophosphates.[1]

Precautions.—It must be borne in mind that the addition of substances to a medium, or yeast extract, may, and frequently does, alter the normal reactions. Furthermore the isolation of a substance that does not appear as an end product does not necessarily indicate that this substance is an intermediate in the main fermentation. Rigorous proofs, established by careful research, are necessary.

Neuberg's Scheme.—Neuberg's scheme[2] for the mechanism of the alcohol fermentation, based partly on the fact that pyruvic acid is an intermediate and is fermented at a rate equal to that of g ucose and on the fact that a triose can be isolated, is as follows: 1 molecule of a hexose is broken down to 2 molecules of a triose ($C_3H_6O_3$), probably a hydrated form of methylglyoxal. The triose, through loss of water, forms methylglyoxal or an active form of this compound. Methylglyoxal, a ketonic aldehyde, is converted to pyruvic acid by oxidation, while another intermediate product is reduced. Pyruvic acid is decomposed to acetaldehyde and carbon dioxide under the influence of the enzyme, carboxylase. Acetaldehyde, in an acid medium, is reduced to ethyl alcohol, while methylglyoxal is being oxidized to pyruvic acid (a simultaneous oxidation-reduction). The scheme thus outlined may be illustrated as follows:

SCHEME I



(Neuberg's "first form" of fermentation)

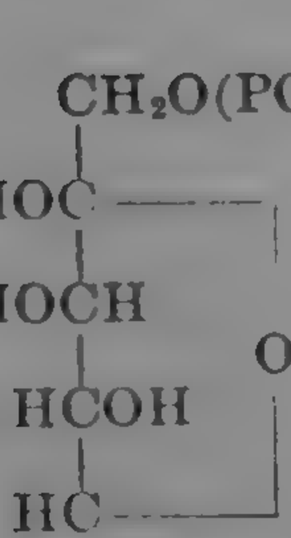
* MICHAELIS, L., V. MORAQUES-GONZALEZ, and C. V. SMYTHE, *Enzymologia*, **3**: 242 (1937).

[1] SMYTHE, C. V., *Jour. Chem.*, **118**: 619 (1937).

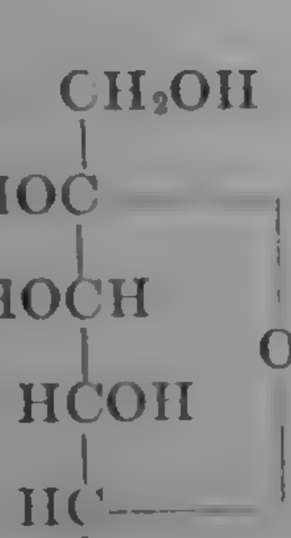
[2] STEPHENSON, *loc. cit.*

TABLE 33.—SOME IMPORTANT INTERMEDIATE AND END PRODUCTS OF THE ETHYL ALCOHOL FERMENTATION

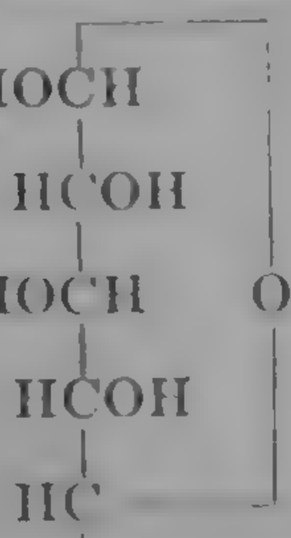
I. *Intermediate Products*



Fructo-furanose 1:6-diphosphate

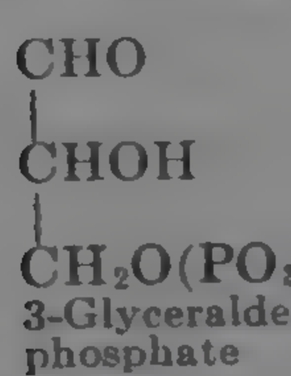


Neuberg ester

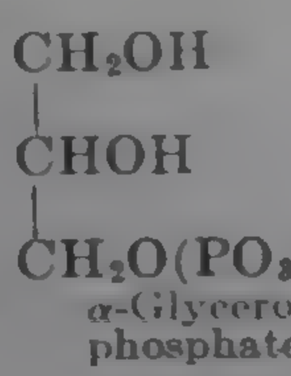


Robison ester

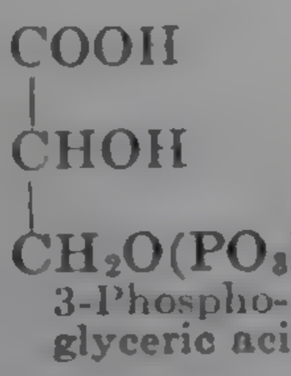
Dihydroxyacetone phosphate



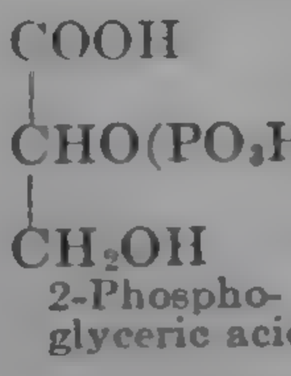
3-Glyceraldehyde phosphate



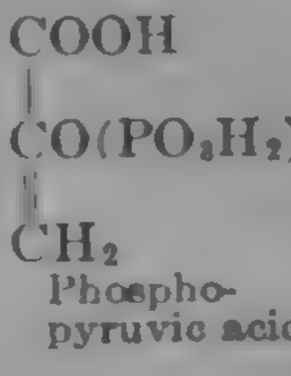
α-Glycerol phosphate



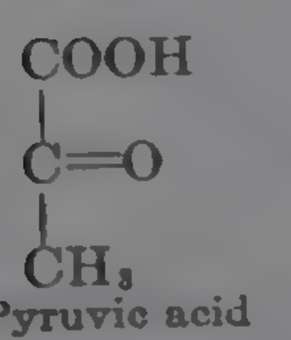
3-Phospho-glyceric acid



2-Phospho-glyceric acid

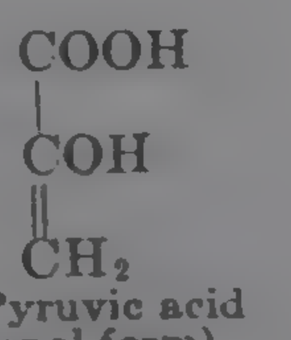


Phospho-pyruvic acid

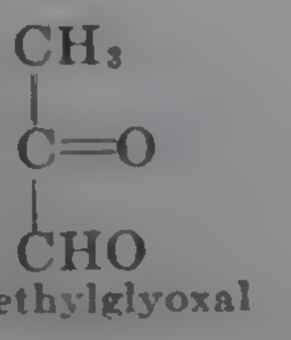


Pyruvic acid

or



Pyruvic acid (enol form)

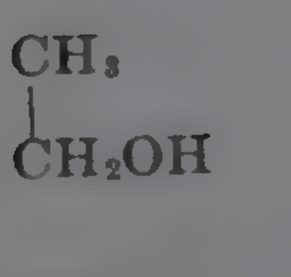


Methylglyoxal

Acetaldehyde

II. *End Products*

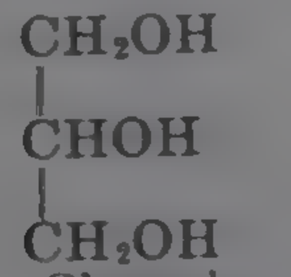
A. Derived from Sugar



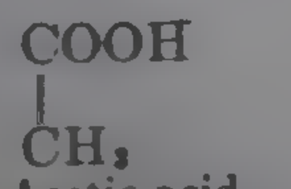
Ethyl alcohol

CO_2

Carbon dioxide

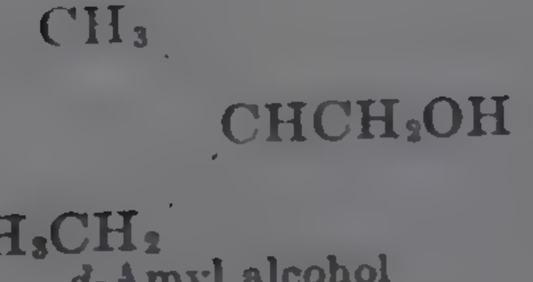


Glycerol

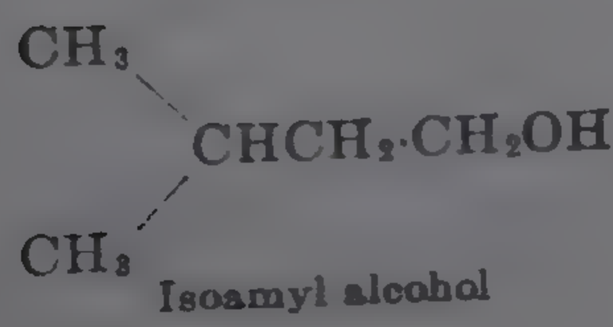


Acetic acid

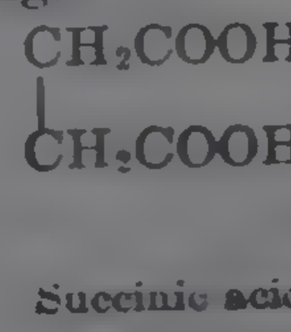
B. Not Derived from Sugar



d-Amyl alcohol



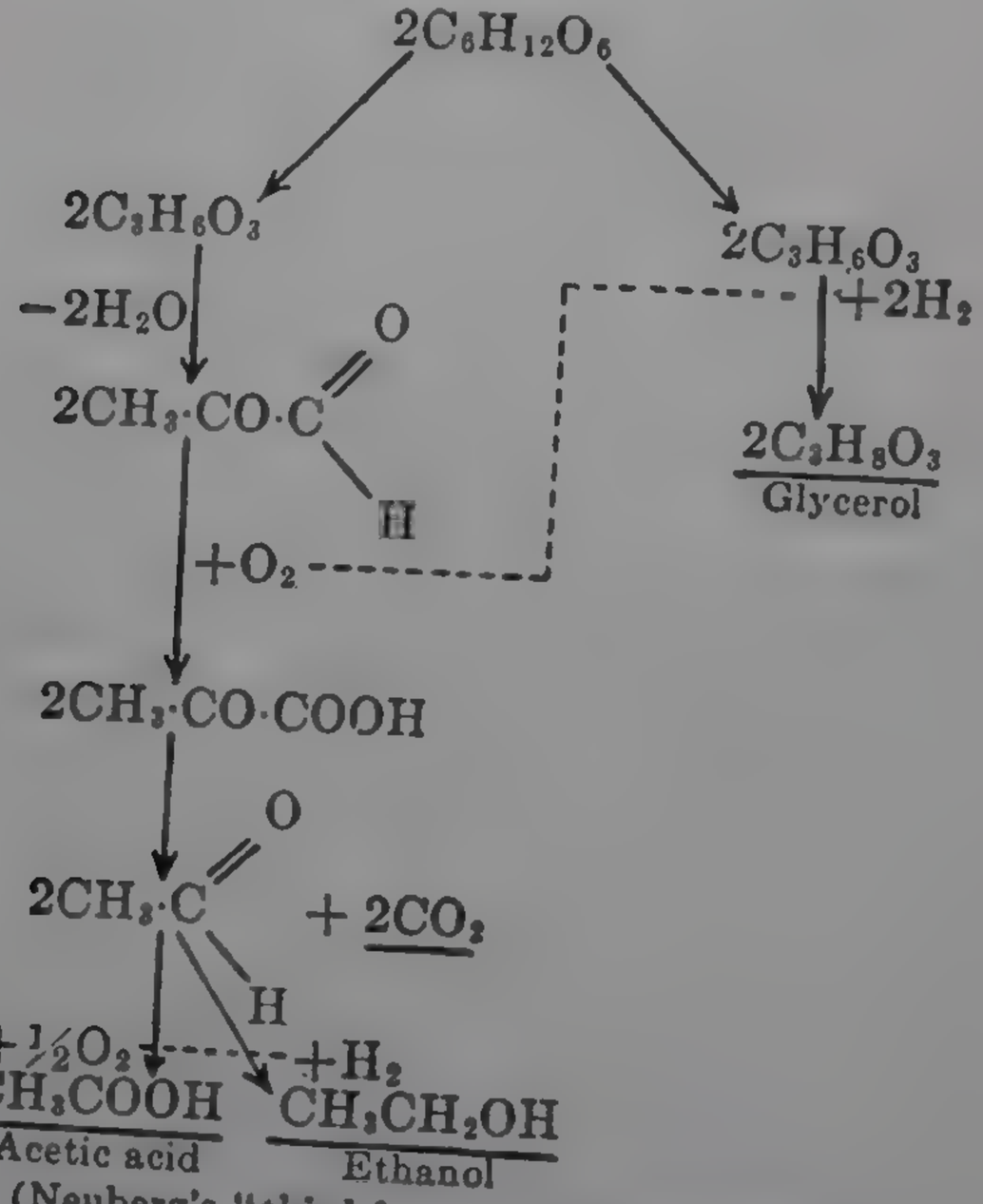
Isoamyl alcohol



Succinic acid

In case the reaction is alkaline, a compound, believed to be unstable and of the formula $C_3H_6O_3$, acts as a hydrogen acceptor together with acetaldehyde. The unstable $C_3H_6O_3$ compound is reduced to glycerol, while acetaldehyde is oxidized in part to acetic acid and reduced in part to ethanol. (A Cannizzaro reaction.)

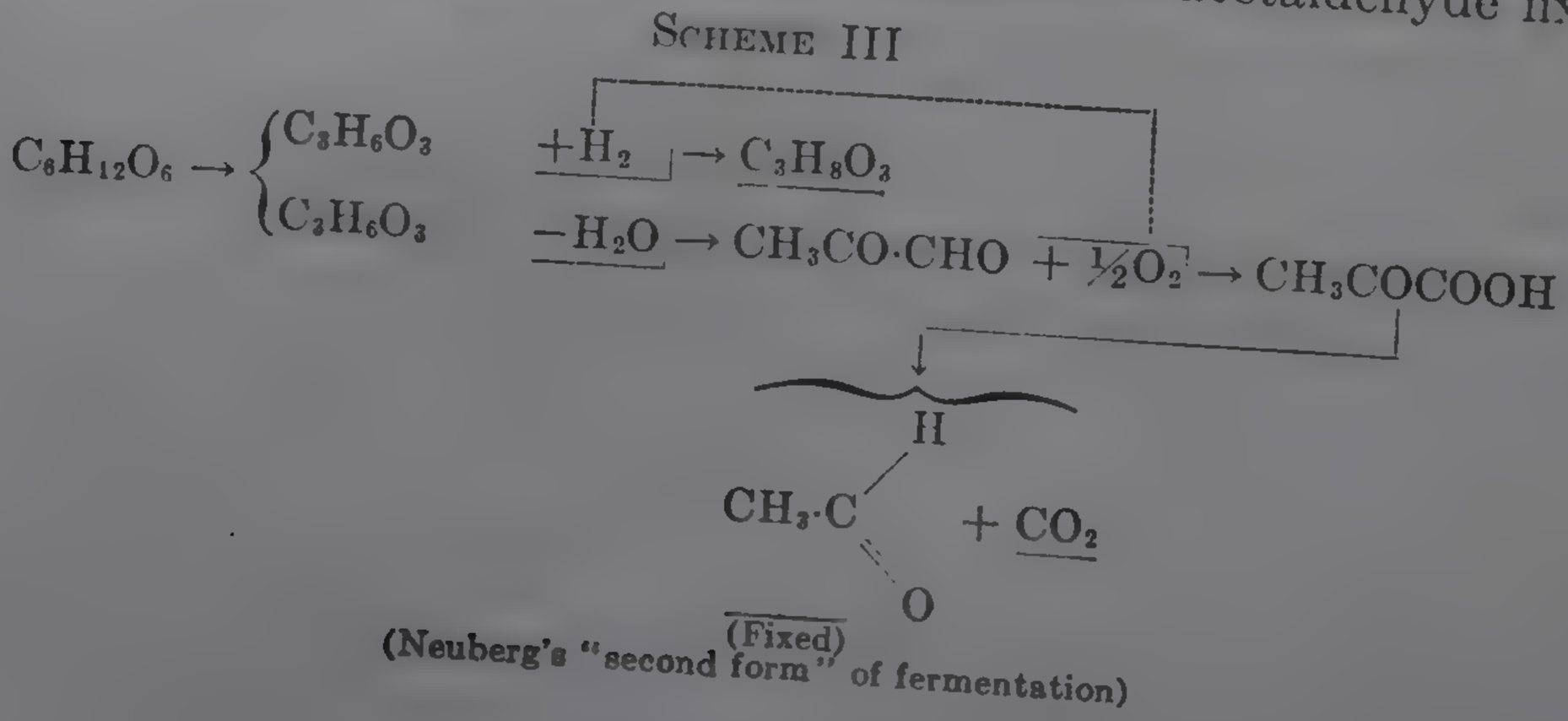
SCHEME II



(Neuberg's "third form" of fermentation)

One method of producing glycerol is by the sulphite fixation process. Acetaldehyde may be fixed by sulphite before it has become reduced or oxidized. The aldehyde thus fixed may be recovered from the fermentation medium and estimated. It is evident that any acetaldehyde that has become fixed is no longer available as a hydrogen acceptor. Acetaldehyde may, then, be replaced in part by $C_3H_6O_3$ as a hydrogen acceptor. $C_3H_6O_3$ is reduced to glycerol in proportion to the amount of acetaldehyde fixed (1 molecule of glycerol for each molecule of acetaldehyde fixed).

SCHEME III

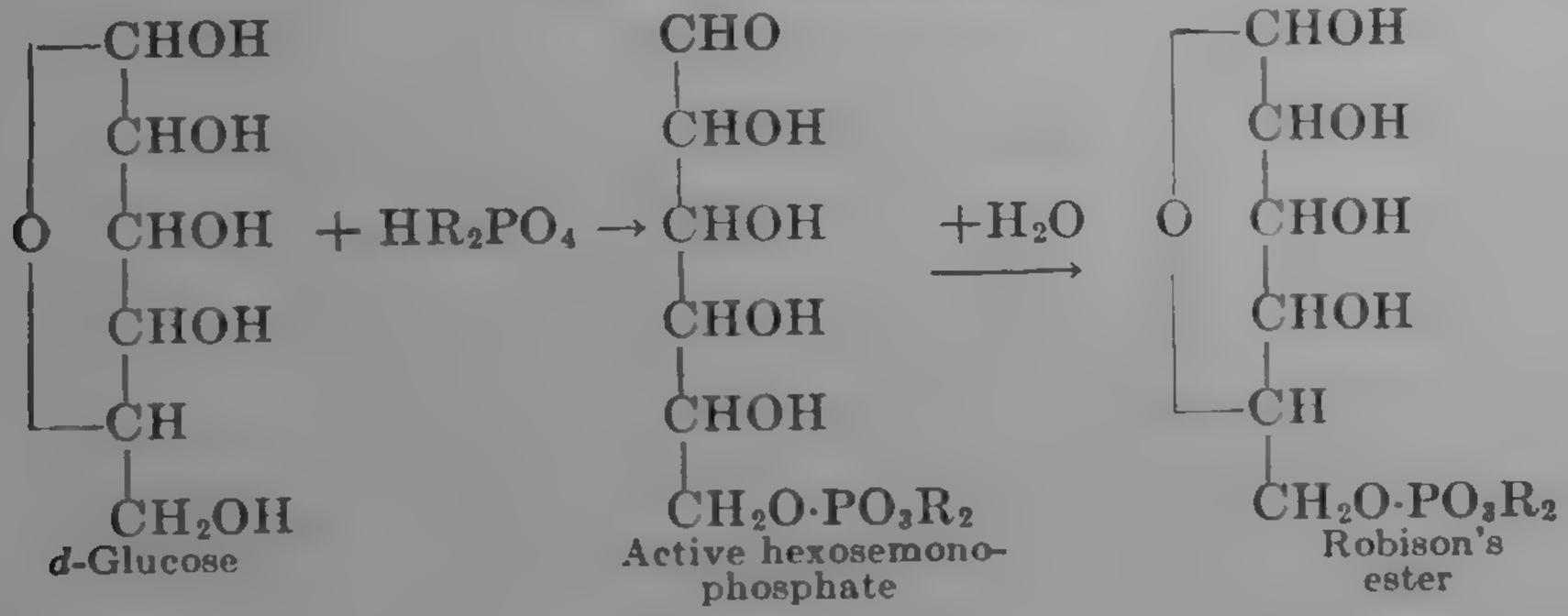


(Neuberg's "second form" of fermentation)

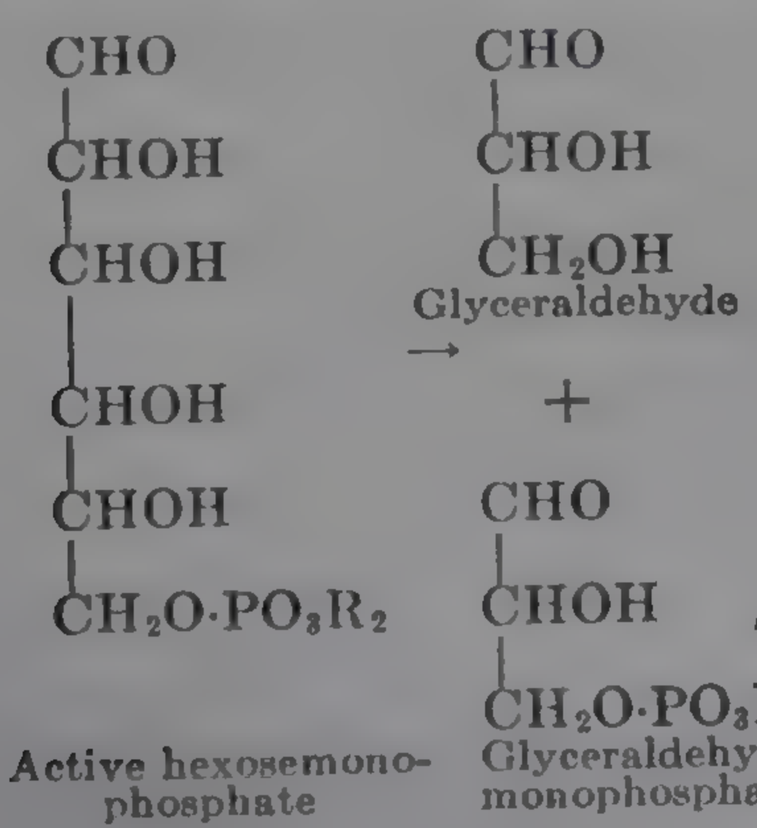
The normal fermentation, according to Neuberg, proceeds chiefly as shown in the first scheme, but some glycerol and acetic acid occur and hence the second scheme is realized in part.

KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE[1]

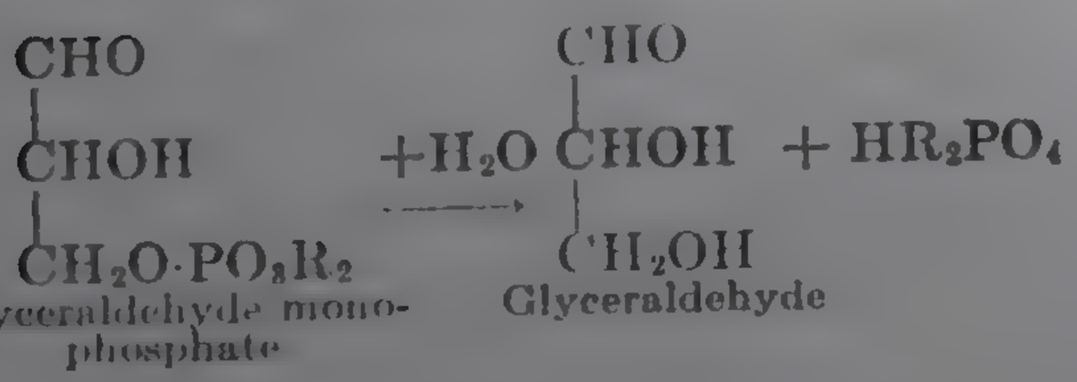
I. *Initial Phosphorylation*



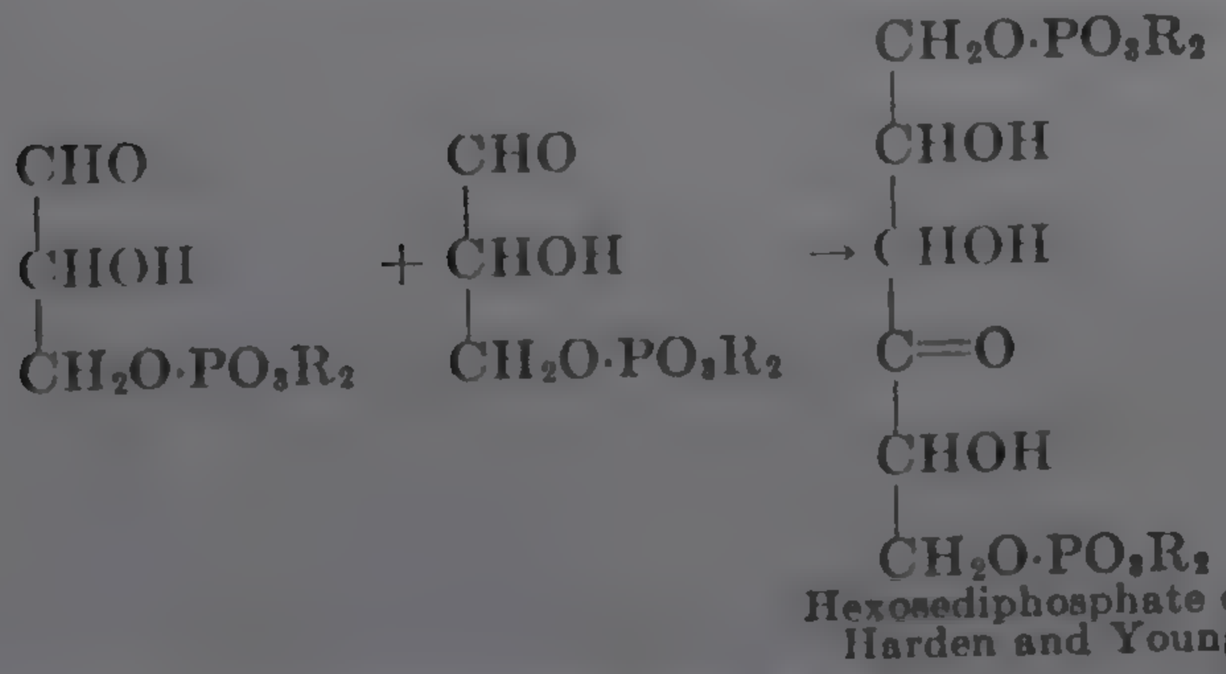
II. *Oxidoreduction of Hexosemonophosphate*



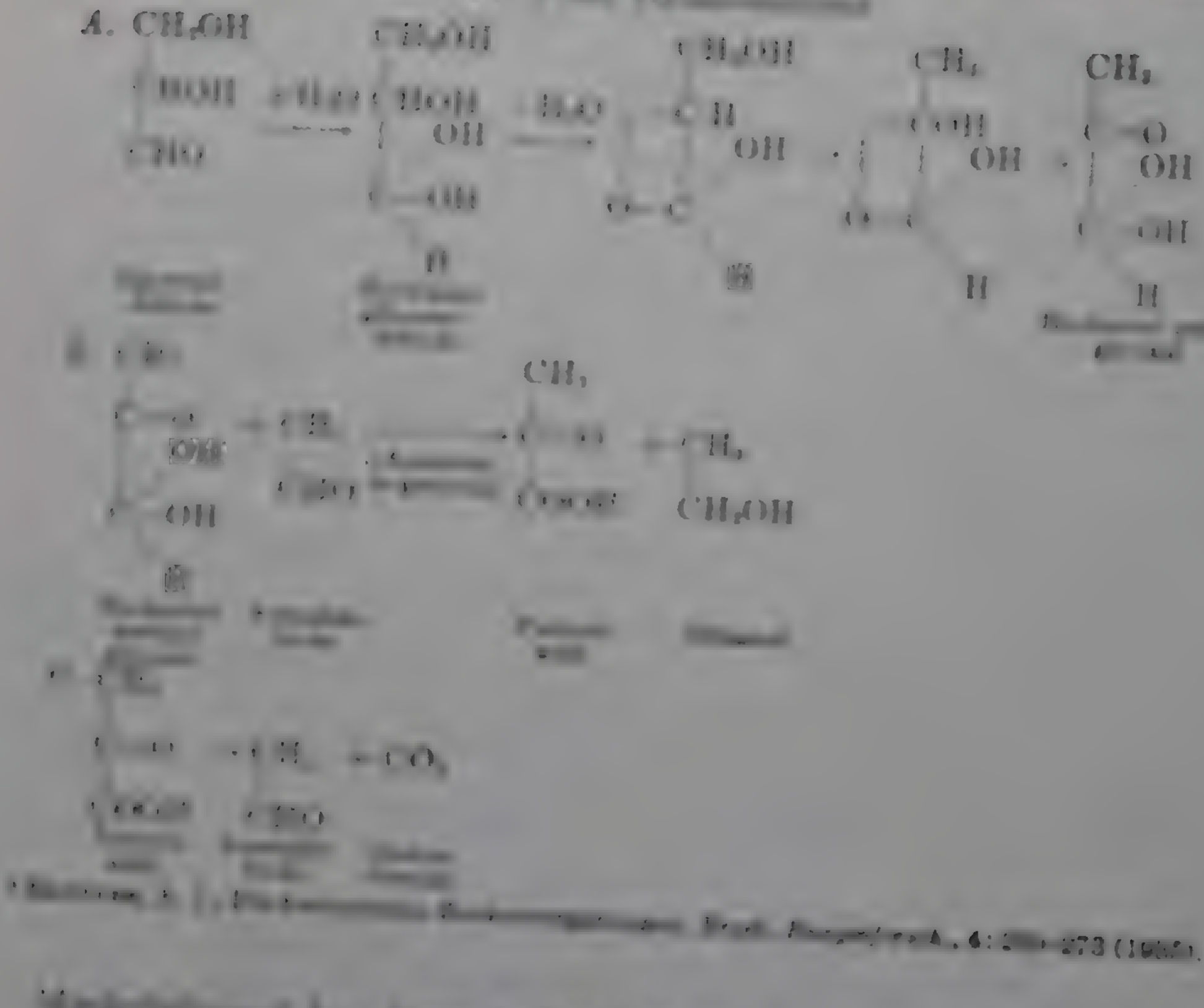
III. *Hydrolysis of Triosephosphate*



Condensation Reaction



[1] KLUYVER, A. J., Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).



[illegible]

hydrogen to a hydrogen acceptor and is changed to pyruvic acid. Acetaldehyde acts as the hydrogen acceptor after it is once formed, being reduced to ethanol, while pyruvic acid is being produced. Pyruvic acid is decarboxylated to acetaldehyde and carbon dioxide. The acetaldehyde reacts with more methylglyoxal hydrate and the production of pyruvic acid, ethanol, acetaldehyde, and carbon dioxide continues.

Kluyver[1] believes that the catalytic agents responsible for the

Fig. 32.—Scheme for fermentation.

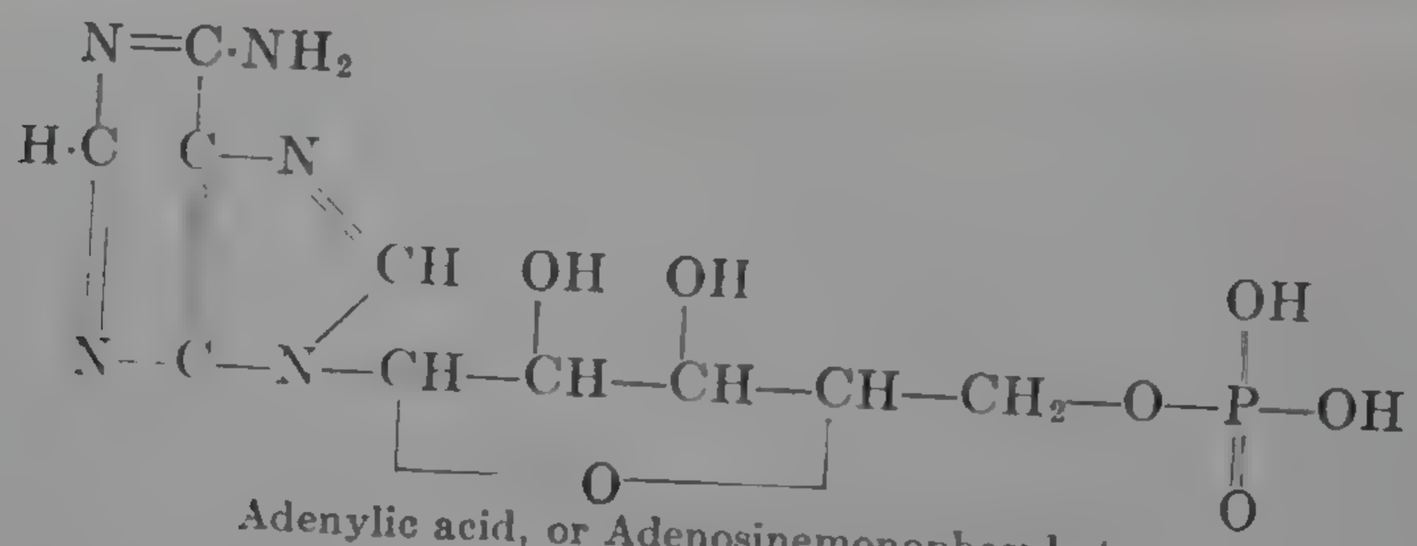
important changes in his scheme for the fermentation of glucose are an oxidoreductase and a phosphatase.

Scheme Based on the Researches of Meyerhof and Others.—In Fig. 32 a scheme for the intermediate reactions occurring in the ethyl alcohol fermentation is shown.

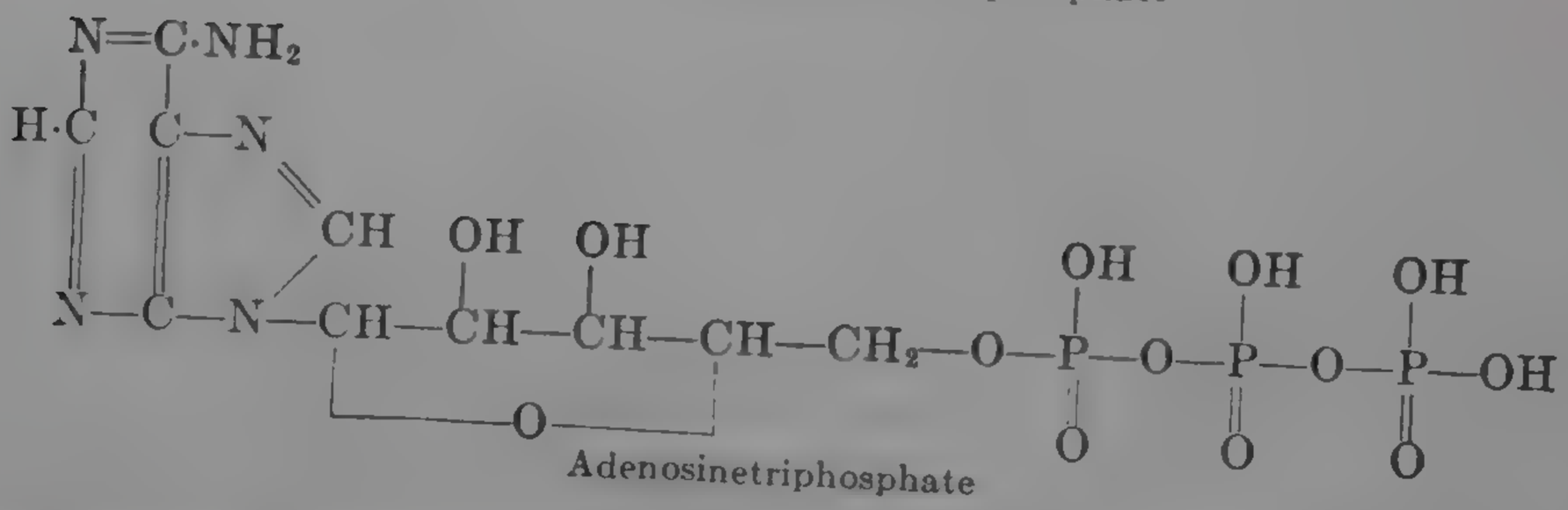
The first step, in the series of reactions, is a phosphorylation of the sugar (glucose, fructose, or mannose) in which a phosphorylating coenzyme, known as the adenylic system, and obtainable in a yeast dialyzate function.[2] This system, or coenzyme, consists of adenylic acid (adenosinemonophosphate), adenosinediphosphate and adenosinetriphosphate. The first two of these compounds have the ability to pick up phosphate, while the third, adenosinetriphosphate, donates phosphate. The formulas for two important members of the adenylic system follow:

[1] For a further discussion of this subject, the reader is referred to A. J. Kluyver, "The Chemical Activity of Microorganisms," University of London Press, Ltd., 1931.

[2] Meyerhof, O., *Nature*, **141**: 855 (1938).



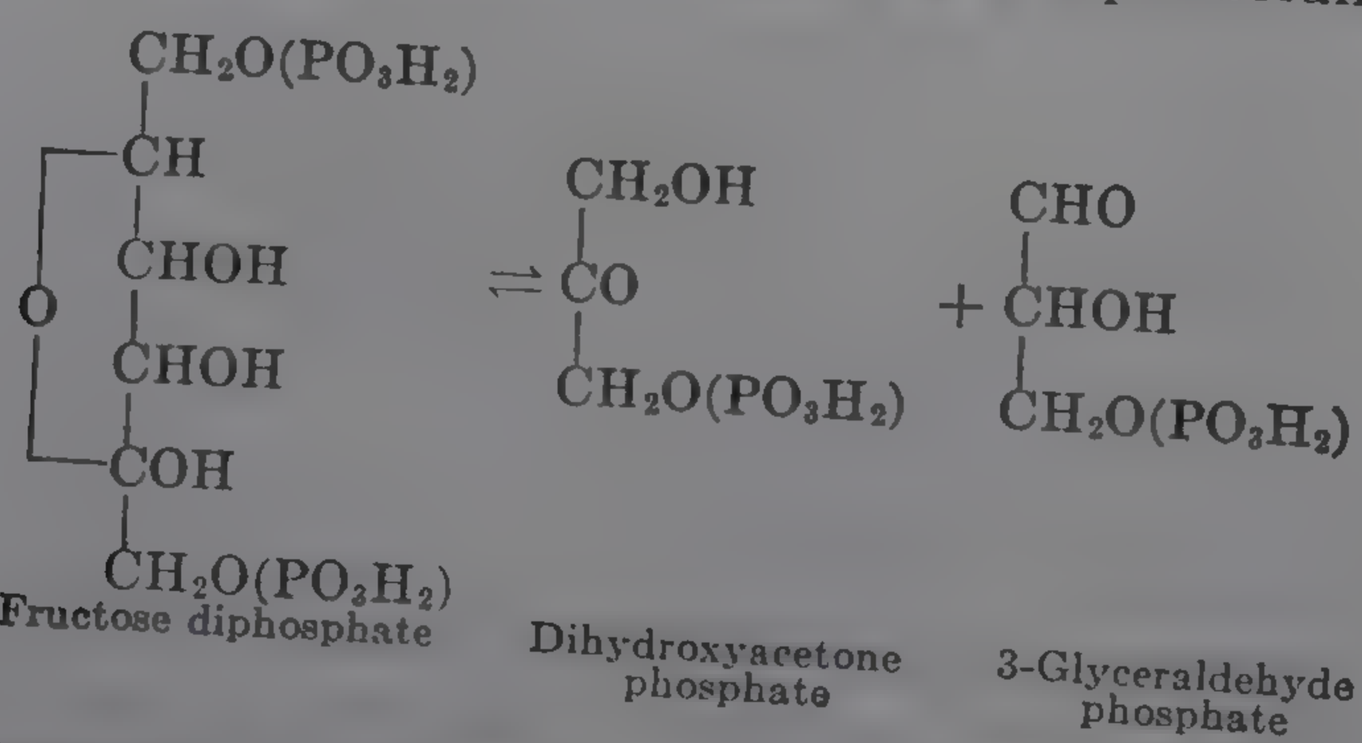
Adenylic acid, or Adenosinemonophosphate



Adenosinetriphosphate

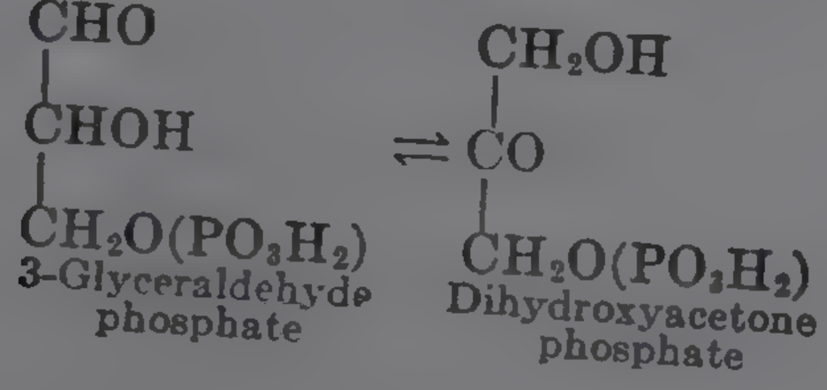
The hexose accepts phosphate from adenosinetriphosphate, the hexosemonophosphate appearing first,[1] then the hexosediphosphate upon further phosphorylation. Once the fermentation has reached the pyruvic acid stage, the phosphate given off by phosphopyruvic acid becomes available for phosphorylating additional hexose.

Fructose diphosphate, or diphosphoric acid (the common diphosphate obtained from glucose, fructose, or mannose), splits to form 2 molecules of triosephosphate with which it is in equilibrium:



Fructose diphosphate — Dihydroxyacetone phosphate — 3-Glyceraldehyde phosphate

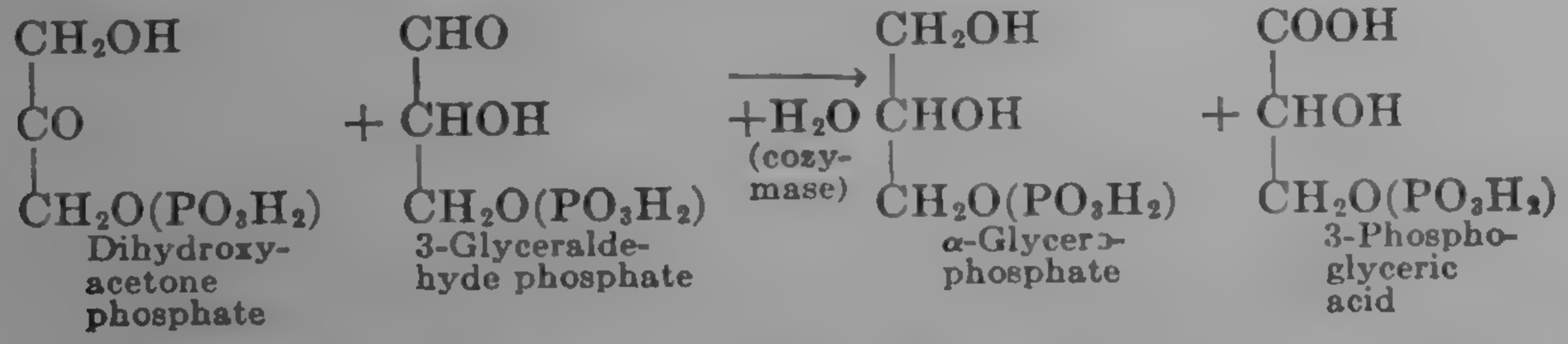
3-Glyceraldehyde is in equilibrium with dihydroxyacetone and is converted largely to the latter, according to Meyerhof.[1]



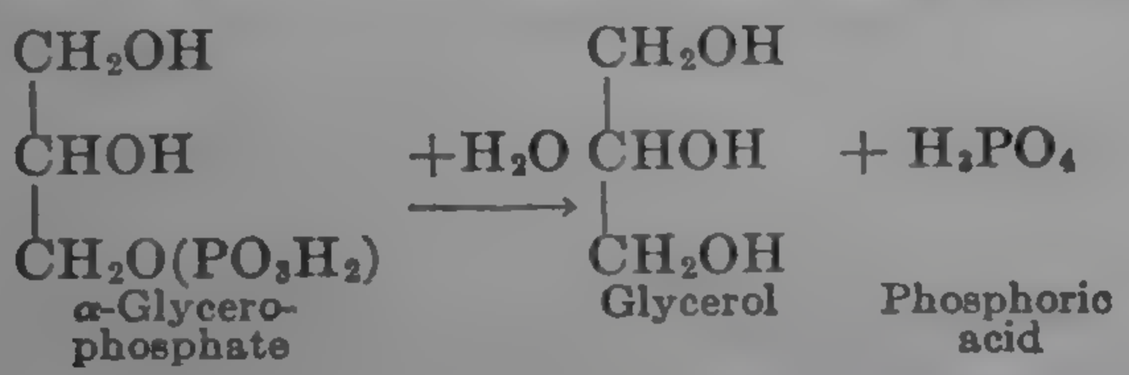
3-Glyceraldehyde phosphate — Dihydroxyacetone phosphate

[1] *Ibid.*

During the initial stages of fermentation (the induction period), before any acetaldehyde is produced, 2 molecules of triosephosphate dismutate to form 1 molecule of 3-phosphoglyceric acid and 1 molecule of α-glycerophosphate, this being an oxidation-reduction reaction in which cozymase (coenzyme I) is active:

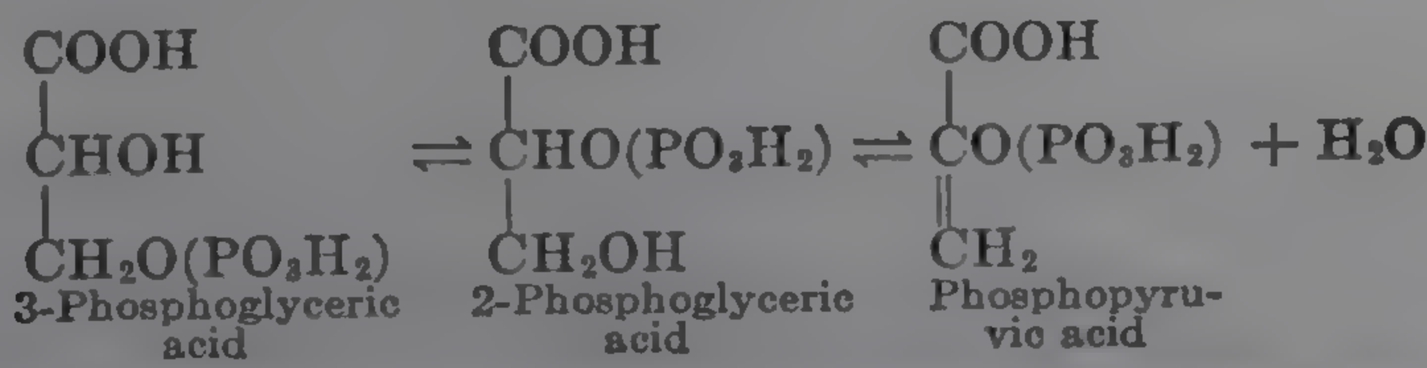


α-Glycerophosphate is hydrolyzed to glycerol and phosphoric acid:



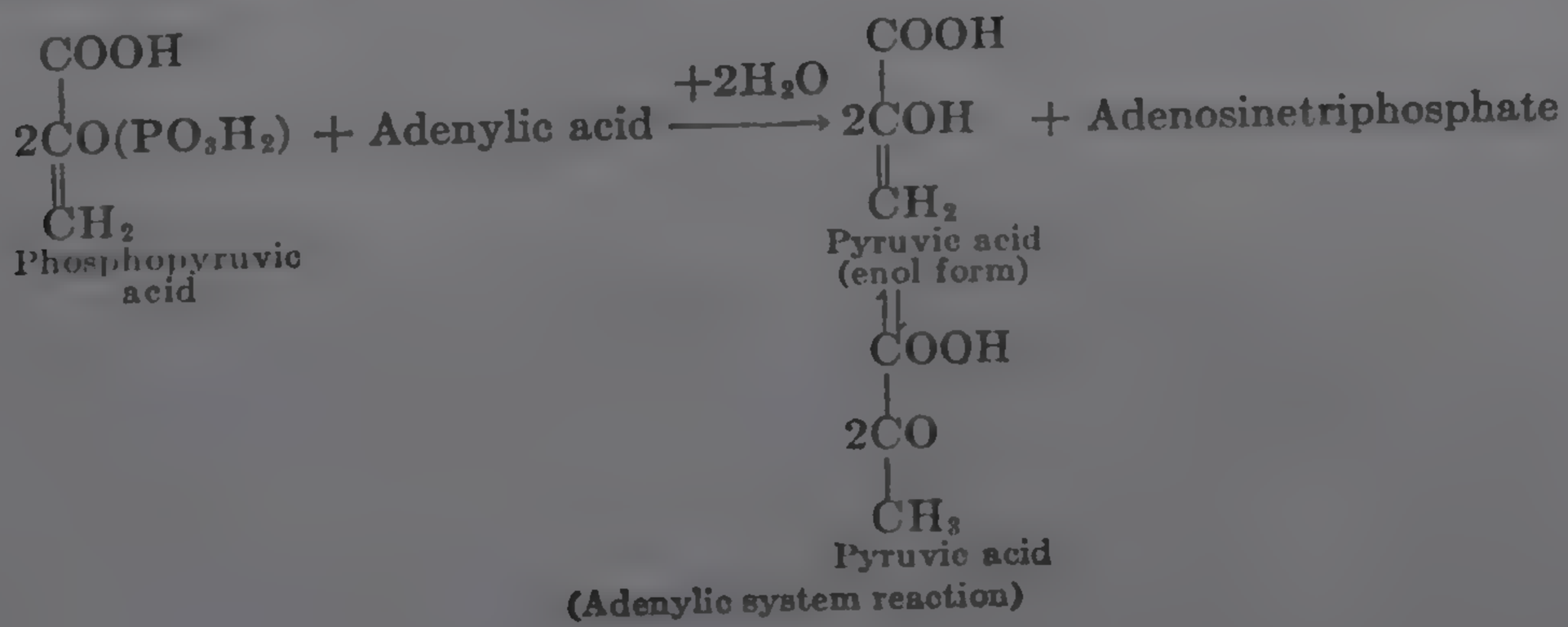
The preceding reaction accounts for the small amount of glycerol that is produced in the normal ethyl alcohol fermentation.

3-Phosphoglyceric acid proceeds, through a series of reversible enzyme reactions, to break down through 2-phosphoglyceric acid to phosphopyruvic acid:



The addition of sodium fluoride to a yeast extract containing either 3-phosphoglyceric acid or 2-phosphoglyceric acid prevents the formation of phosphopyruvic acid, for the enzyme (enolase) is poisoned.

Phosphopyruvic acid is dephosphorylated by adenylic acid to form pyruvic acid, the adenylic acid taking up phosphate to become adenosinetriphosphate.



Adenosinetriphosphate donates phosphate to 2 molecules of hexose to form 2 molecules of hexosemonophosphate and 1 molecule of adenylic acid, the net result being expressed by the following reaction:

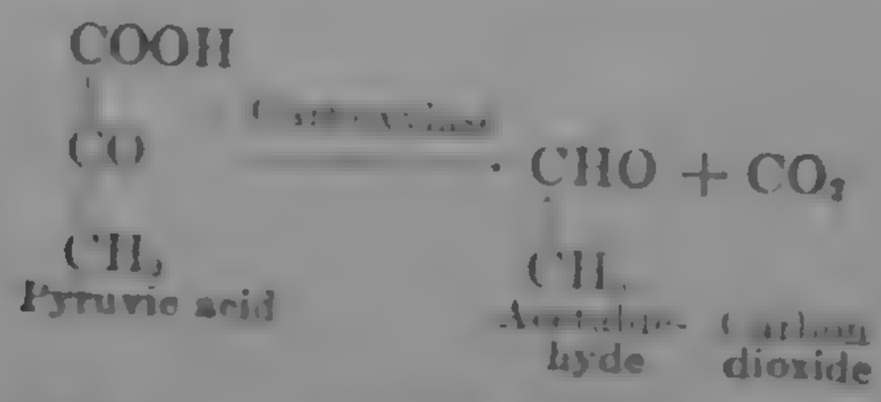
Phosphopyruvic acid + Hexose → Pyruvic acid + Hexosemonophosphate

Additional phosphopyruvic acid is broken down in the same manner with the result that the hexosemonophosphate is further phosphorylated:

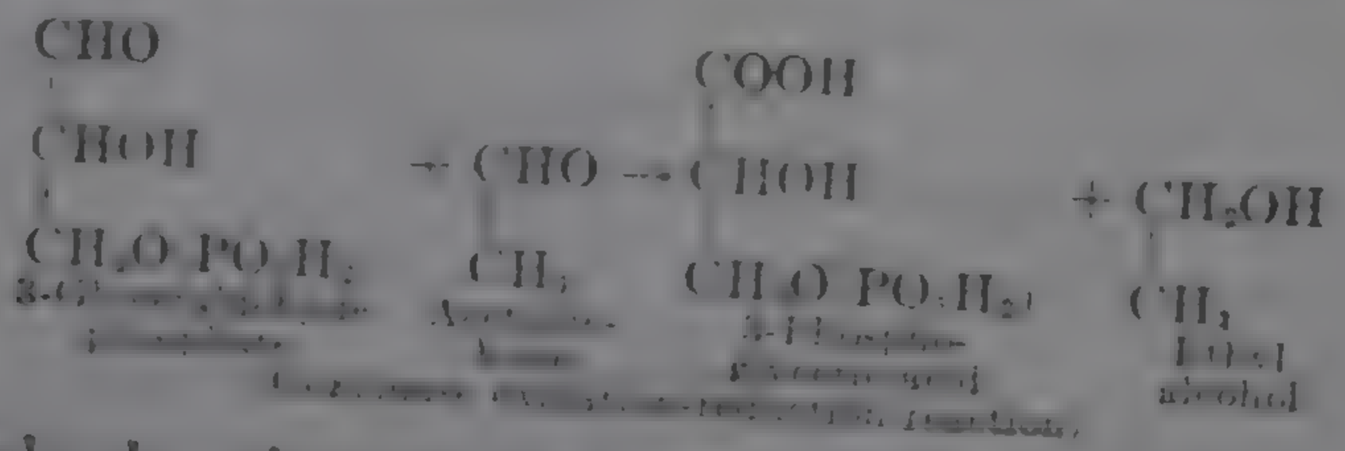
Phosphopyruvic acid + Hexosemonophosphate → Pyruvic acid + Hexosediphosphate

In this manner, hexosediphosphate arises during the stationary phase of fermentation.

Pyruvic acid is broken down to acetaldehyde and carbon dioxide by the enzyme carboxylase:



When present in sufficient quantity, acetaldehyde reacts with reduced cozymase (coenzyme I) with the result that acetaldehyde is reduced to ethyl alcohol while cozymase is oxidized. The oxidized cozymase now reacts with the triosephosphate (glyceraldehyde phosphate), oxidizing it to 3-phosphoglyceric acid, while cozymase becomes reduced again. It is thus seen that cozymase acts in the capacity of a hydrogen carrier in this oxidation-reduction. This reaction occurs normally during the stationary phase of fermentation.



The phosphoglyceric acid thus formed is broken down through the series of reactions outlined above to produce more acetaldehyde, which reacts with more triosephosphate to produce more phosphoglyceric acid. The cycle continues in this manner to the end of the fermentation.

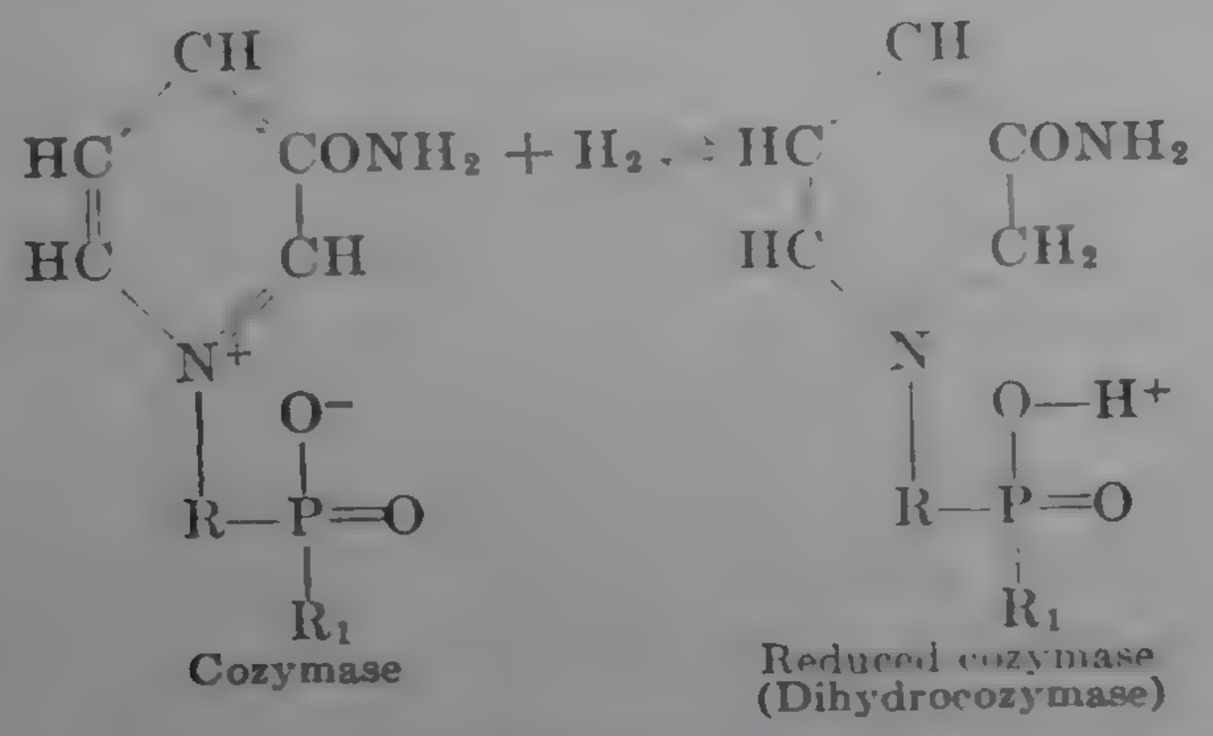
Types of Reactions.—Meyerhof recognizes four main types of reactions in the foregoing scheme for the intermediate reactions of the ethyl alcohol fermentation. These include the phosphorylation-dephosphorylation reactions, the oxidation-reduction reactions, the reversible reactions, and the decarboxylation reaction.

The adenylic system, the mechanism of which has already been

explained, is concerned in the phosphorylation-dephosphorylation reactions.

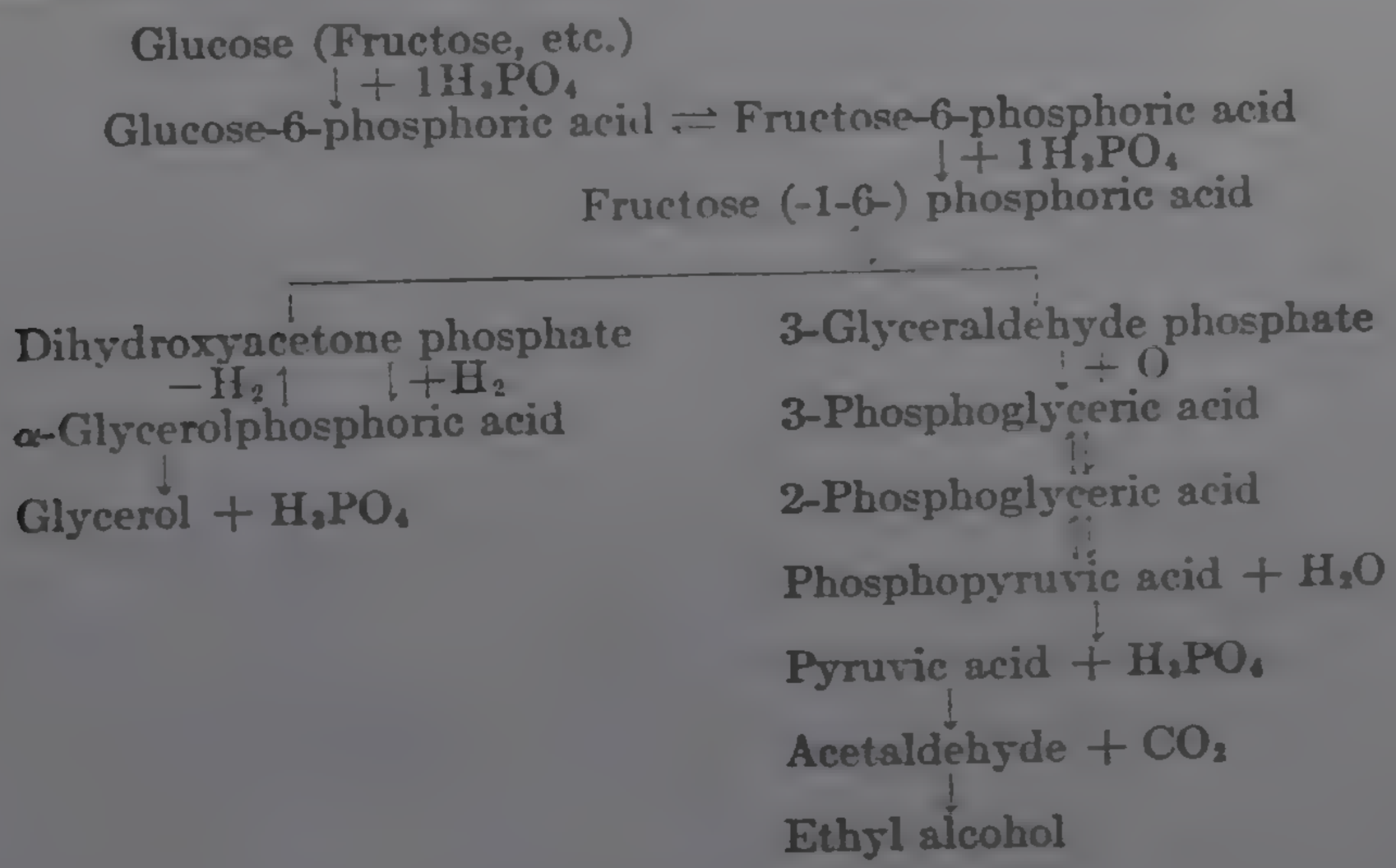
Oxidoreductases and cozymase (coenzyme I) are responsible for the conversion of the triose phosphates to α-glycerophosphoric acid and 3-phosphoglyceric acid during the induction period and for the oxidation of triosephosphate (glyceraldehyde phosphate) with the simultaneous reduction of acetaldehyde during the stationary period of fermentation.

Nicotinic acid amide[1] is the hydrogen-carrying group of cozymase. The function of cozymase in transporting hydrogen may be illustrated by the following equation in which R represents the ribose phosphoric acid group of cozymase (see structural formula on p. 41) and R_1 represents the adenosinemonophosphate group:



The principal reversible reactions include the reaction between hexosediphosphate and 2 molecules of triosephosphate; the reaction between dihydroxyacetone phosphate and glyceraldehyde phosphate; and the reactions between 3-phosphoglyceric acid, 2-phosphoglyceric acid, and phosphopyruvic acid.

A summary of the reversible reactions appears in the following scheme of Meyerhof.[1] The relation of each intermediate or end product to its immediate predecessor is indicated.



[1] *Ibid.*

Some Enzymes Active during Fermentation.—Several enzymes catalyze the intermediate reactions of the ethyl alcohol fermentation. Hexokinase converts fermentable hexoses to more active forms. Zymohexase catalyzes the breakdown of hexosediphosphate to triosephosphates. Oxidoreductase (mutase, dehydrase) with cozymase plays a very important role in the oxidation-reduction reactions. The conversion of phosphopyruvic acid to pyruvic and phosphoric acids is catalyzed by enolase, while carboxylase breaks down pyruvic acid to form acetaldehyde and carbon dioxide.

In Table 34 (Cori, 1942) are shown reactions catalyzed by 12 separate enzymes and dialyzable components required for the action of some of these enzymes.

TABLE 34.—ALCOHOLIC FERMENTATION[1]

(*Intact yeast cell:* glucose = $2CO_2$ + 2 alcohol; *cell-free juice:* 2 glucose + $2KH_2PO_4$ = $2CO_2$ + 2 alcohol + 1 hexosediphosphate)

Non-dialyzable enzymes and reactions catalyzed	Dialyzable components
1. Glucose + ATP → glucose-6-P + ADP*	*Mg++ ions needed for reaction
2. Glucose-6-P ⇌ fructose-6-P	ATP = Adenosinetriphosphate
3. Fructose-6P + ATP → fructose1-6-diP + ADP*	ADP = Adenosinediphosphate
4. Fructose-diP ⇌ dihydroxyacetone-P + glyceraldehyde-P	
5. Dihydroxyacetone-P ⇌ glyceraldehyde-P	
6. 3-glyceraldehyde-P + KH_2PO_4 + Pyr. ⇌ 1-3-glyceric acid-P + H_2Pyr.[2]	Pyr. = Pyridine-nucleotide H_2Pyr. = Reduced pyridine-nucleotide KH_2PO_4 = Inorganic phosphate
7. 1-3-glyceric acid-P + ADP ⇌ 3-glyceric acid-P + ATP*	
8. 3-glyceric acid-P ⇌ 2-glyceric acid-P*	
9. 2-glyceric acid-P ⇌ pyruvic acid-P + H_2O*[2]	
10. Pyruvic acid-P + ADP → pyruvic acid + ATP*	
11. Pyruvic acid + DPT → acetaldehyde + CO_2	DPT = Diphosphothiamin
12. Acetaldehyde + H_2Pyr. ⇌ alcohol + Pyr[2]	

NOTE: Owing to the splitting of the sugar molecules in two halves in reaction 4, reactions 6 to 12 should be multiplied by two to balance the equation given above for intact yeast cells.

[1] CORI, C. F., *Proc. 5th Ann. Meet. Am. Soc. Brewing Chemists*, May 25–27, 1942.

[2] Enzymes obtained in crystalline form.

Basis for Theory.—Space permits only a relatively brief discussion of the facts upon which the foregoing theory is based. Many of the important discoveries were made during investigations of the intermediate reactions concerned in the formation of lactic acid in muscle

extract and were subsequently established for the ethyl alcohol fermentation.

Parnas, Lutwak-Mann, and Mann have demonstrated the importance of adenylic acid and adenosinetriphosphate as dephosphorylating and phosphorylating agents, respectively.

Hexosediphosphate has been isolated repeatedly and is readily fermented by yeast and muscle extracts.

Hexosediphosphate enters into equilibrium with the 2 molecules of triosephosphate (Meyerhof and Lohmann, 1934): dihydroxyacetone phosphate, synthesized by Kiessling, and 3-glyceraldehyde phosphate, synthesized by H. O. L. Fischer (1932). These two triosephosphates have been isolated from muscle extract and subsequently from yeast extract fermentations of glucose or of hexosediphosphate with the aid of monoiodoacetic acid. They are readily fermented by yeast extract and together form an equilibrium in which dihydroxyacetone phosphate predominates. Whether one starts with a mixture of synthetic or natural triosephosphates or with hexosediphosphate a reversible reaction quickly occurs between hexosediphosphate and the triosephosphates in the presence of yeast extract.

Important information concerning the 3-carbon intermediates has been obtained by the use of sodium fluoride and monoiodoacetic acid. Embden (1933), using a muscle extract poisoned with sodium fluoride, showed that phosphoglyceric acid was an intermediate compound. He showed that in the absence of fluoride, phosphoglyceric acid was converted to pyruvic and phosphoric acid through enzymic action. Nilsson isolated phosphoglyceric acid from a fermentation by yeast extract.

Meyerhof and Kiessling showed that there were two phosphoglyceric acids: 3-phosphoglyceric acid and 2-phosphoglyceric acid. These acids have been isolated from muscle extract and synthesized by Kiessling. They differ in structure, solubilities, and optical rotations.

Phosphopyruvic acid was isolated by K. Lohmann as a crystalline salt. It has been synthesized by Kiessling.

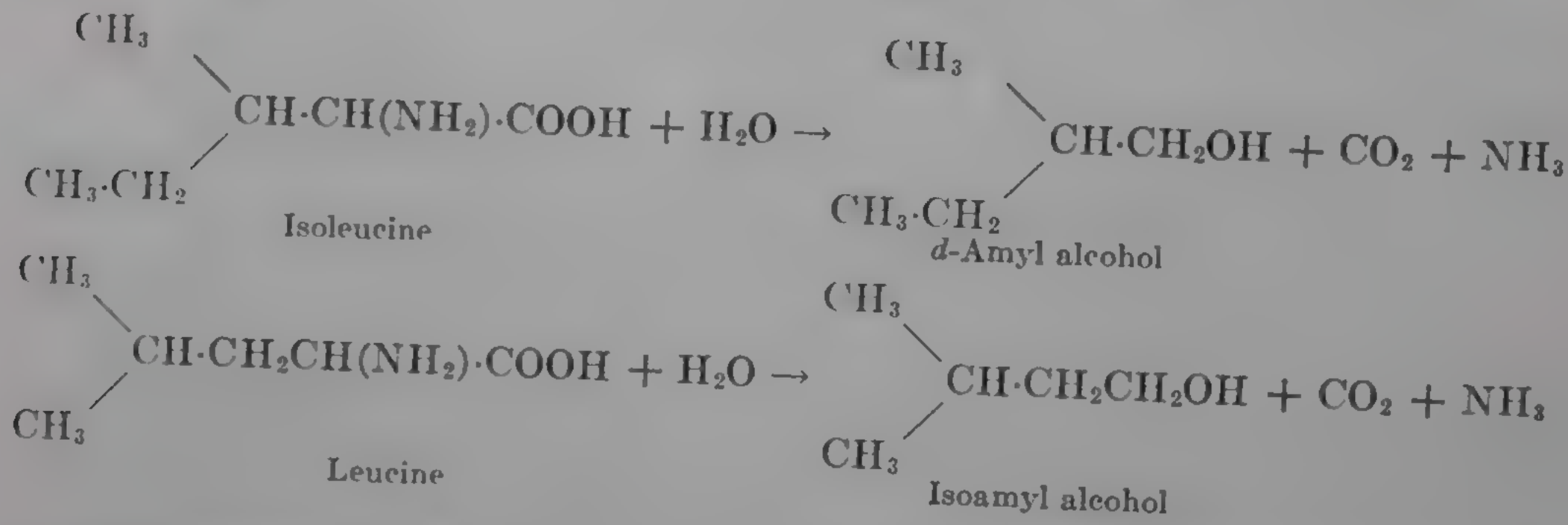
Pyruvic acid has been isolated as an intermediate and is readily fermented by living yeasts or yeast extracts. It is converted to acetaldehyde and carbon dioxide by the enzyme carboxylase, discovered by Neuberg.

Acetaldehyde has been demonstrated by fixation with sulphites and dimedon.

The Origin of Amyl and Isoamyl Alcohols.—Ehrlich[1] has shown that amyl and isoamyl alcohols are derived from the amino acids, namely,

[1] HARDEN, A., "Alcoholic Fermentation," 4th ed., Longmans, Green & Company, New York, 1932.

isoleucine and leucine, respectively. These acids are obtained from the medium usually, but in cases of nitrogen deficiency may originate from yeast protein.



The formation of these alcohols from their corresponding amino acids may be demonstrated by inoculating sterile mashes, containing a fermentable sugar and a measured quantity of one or both of the acids, with a pure culture of yeast and analyzing the mashes after the fermentation for the amino acids and alcohols. Control experiments should be carried out at the same time, of course, with mashes that do not contain added amino acids.

The ammonia set free in the foregoing reactions is utilized immediately by the yeast cells.

The relative proportions and likewise the quantities of the two alcohols formed depend on the composition of the medium, *i.e.*, on the relative amounts of the two amino acids, the presence or absence of ammonium salts, and the nature of the sugar; on the species of yeast; on the nutritive condition of the yeast; and on other factors. Certain ammonium salts cause a diminution in the normal yield of amyl alcohols, ammonia being derived from the salts rather than from the amino acids.

Leucine and isoleucine are not converted to their corresponding alcohols by living yeast cells in the absence of a fermentable sugar, nor are they converted by yeast juice or by zymin (a product prepared by treating yeast with alcohol and ether, or by acetone and ether).

Alcohols are produced from other alpha-amino acids in a similar manner by yeasts. Such products are believed to contribute to the flavor of beer, wine, rum, and other alcoholic beverages. For example, tyrosol, the alcohol produced from tyrosine, possesses a bitter taste and is concerned in the flavor of beer.

Succinic Acid (HOOC·CH$_2$·CH$_2$·COOH).—Succinic acid is believed to arise from glutamic acid during fermentation. Ehrlich discovered (1909) after experimentation with several amino acids, which included aspartic acid (HOOC·CH$_2$·CH(NH$_2$)·COOH), that glutamic acid was

the only amino acid added to the fermentation medium that produced a well-defined increase in the quantity of succinic acid.

The probable course of its formation from glutamic acid is as follows:[1]

$$\begin{array}{l} \mathrm{HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH} \quad \text{(Glutamic acid)} \\ \downarrow \tfrac{1}{2}\mathrm{O_2} \quad \text{(Oxidative deamination)} \\ \mathrm{NH_3 + HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH} \quad (\alpha\text{-Ketoglutaric acid}) \\ \downarrow \quad \text{(Decarboxylation)} \\ \mathrm{CO_2 + HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CHO} \quad \text{(Succinic semialdehyde)} \\ \downarrow \tfrac{1}{2}\mathrm{O_2} \quad \text{(Oxidation)} \\ \mathrm{HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} \quad \text{(Succinic acid)} \end{array}$$

In the absence of added nitrogen-containing substances, succinic acid may be produced from the glutamic acid derived from the autolysis of the protein of yeast.

Succinic acid is not formed by yeast in the absence of sugar nor is it produced by yeast juice or zymin.

References on the Mechanism of the Ethyl Alcohol Fermentation

ANDERSON, C. G.: "An Introduction to Bacteriological Chemistry," William Wood & Company, The Williams & Wilkins Company, Baltimore, 1938.

ANHAGEN, E.: Co-Carboxylase, ein neues Co-Enzym der alkoholischen Gärung, *Zeit. physiol. Chem.*, **204**: 149 (1932).

CORI, C. F.: The Enzymatic Reactions in Alcoholic Fermentation, *Proc. 5th Ann. Meeting Am. Soc. Brewing Chem.*, May 25–27, 1942.

EMBDEN, G., H. J. DEUTICKE, und G. Graft: Über die intermediären Vorgänge bei der Glykolyse in der Muskulatur, *Klin. Wochschr.*, **12**: 213 (1932).

EULER, H. VON: Neuere Ergebnisse an enzymatischen Oxydations- und Reduktions-Systemen, *Ergeb. Enzymforsch.*, **3**: 135 (1934).

——— und K. MYRBÄCK: Co-Zymase und Adenylsäure, *Zeit. physiol. Chem.*, **199**: 189 (1931).

——— und R. NILSSON: Adenosintriphosphorsäure und Co-Zymase, *Zeit. physiol. Chem.*, **208**: 173 (1932).

FISCHER, H. O. L., und E. BAER: Über die 3-Glycerinaldehyde-phosphorsäure, *Ber.*, **65**: 337 (1932).

FREY, C. N.: History and Development of Modern Yeast Industry, *Ind. Eng. Chem.*, **22**: 1154 (1930).

GODA, T.: Beitrag zur enzymatischen Oxydoreduktion zwischen Glycerinphosphorsäure und Brenztraubensäure, *Biochem. Zeit.*, **297**: 317 (1938).

———: Kann lebende Hefe Hexosen direkt vergären? *Biochem. Zeit.*, **298**: 431 (1938).

HARDEN, A.: "Alcoholic Fermentation," 4th ed., Longmans, Green & Company, New York, 1932.

——— and R. ROBISON: A New Phosphoric Ester Obtained by the Aid of Yeast-juice (Preliminary note), *Proc. Chem. Soc.*, **30**: 16 (1914).

——— and W. J. YOUNG: The Influence of Phosphates on the Fermentation of Glycose by Yeast Juice (Preliminary communication), *Proc. Chem. Soc.*, **21**: 189 (1905).

[1] *Ibid.*

HOPKINS, R. H., and C. KRAUSE: "Biochemistry Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1937.
KIESSLING, W.: Über die Synthese der Dioxy-aceton-phosphorsäure. *Ber.*, **67**: 869 (1934).
———: Über die Reindarstellung von Glucose-1-phosphorsäure (Cori-Ester), *Biochem. Zeit.*, **298**: 421 (1938).
——— und O. MEYERHOF: Über ein Adenindinucleotid der Hefe: Di-(Adenosin-5'-phosphorsäure), *Biochem. Zeit.*, **298**: 410 (1938).
KLUYVER, A. J.: "The Chemical Activities of Micro-organisms," University of London Press, London, 1931.
———: Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230 (1935).
LEBEDEV, A.: Sur l'extraction de la zymase, *Compt. rend.*, **152**: 1129 (1911).
LEHMANN, J.: Aktivierung von Hefealkoholdehydrogenase durch Co-Enzym, *Biochem. Zeit.*, **272**: 95 (1934).
———: Zur Kenntnis biologischer Oxydations-Reduktions-potentiale. Vorläufige Potentialmessungen im System. Alkohol-Dehydrogenase-Acetaldehyd, *Biochem. Zeit.*, **274**: 321 (1934).
———: Über den Mechanismus der Vergärung des Dioxyacetons, *Biochem. Zeit.*, **277**: 261–267 (1935).
LEVENE, P. A., and A. L. RAYMOND: Hexosemonophosphates. Glucose-3-Phosphate, Glucose-6-Phosphate and Their Bearing on the Structure of Robison's Ester, *Jour. Biol. Chem.*, **89**: 479 (1930).
——— and ———: Hexosemonophosphate (Robison) Natural and Synthetic, *Jour. Biol. Chem.*, **91**: 751 (1931).
——— and ———: Hexosemonophosphates. Synthetic Robison Ester, *Jour. Biol. Chem.*, **92**: 757 (1931).
——— and ———: Hexosemonophosphates. Galactose-6-Phosphate, *Jour. Biol. Chem.*, **92**: 765 (1931).
LOHMANN, K.: Über die Isolierung verschiedener natürlicher Phosphorsäureverbindungen und die Frage ihrer Einheitlichkeit, *Biochem. Zeit.*, **294**: 306 (1928).
——— und P. SCHUSTER: Untersuchungen über die Co-carboxylase, *Biochem. Zeit.*, **294**: 188 (1937).
——— und ———: Über die Co-carboxylase, *Naturwissenschaften*, **25**: 26 (1937).
LUTWAK-MANN, C., und T. MANN: Über die Verkettung der chemischen Umsetzungen in der alkoholischen Gärung, I, *Biochem. Zeit.*, **281**: 140 (1935).
MEYERHOF, O.: Intermediate Products and the Last Stages of Carbohydrate Breakdown in the Metabolism of the Muscle and in Alcohol Fermentation, *Nature*, **132**: 337, 373 (1933).
———: Sur les processus intermédiaires dans la dégradation des glucides (formation d'acid lactique et fermentation alcoolique), *Ann. Inst. Pasteur*, **53**: 221 (1934).
———: Über die Intermediärvorgänge bei der biologischen Kohlehydratspaltung, *Ergeb. Enzymforsch.*, **4**: 208 (1935).
———: The Intermediary Reactions of Fermentation, *Nature*, **141**: 855–858 (1938).
———: Intermediate Reactions of Fermentation, *Wallerstein Labs. Communs.*, **5** (No. 16): 181–186 (1942).
———: New Chemical and Physical Tools for Investigating the Intermediary Metabolism of Carbohydrate, *Wallerstein Labs. Communs.*, **6** (No. 17): 19–25 (1943).
———: Enzymatic Mechanisms of Fermentation, *Am. Brewer*, January, 1943.
———: The Origin of the Reaction of Harden and Young in Cell-free Alcoholic Fermentation, *Jour. Biol. Chem.*, **157** (No. 1): 105–119 (1945).

———: New Investigations in the Kinetics of Cell-free Alcoholic Fermentation. *Antonie Van Leeuwenhoek*, **12**: 140–144 (1947) (Jubilee Vol. A. J. Kluyver).
——— und W. Kiessling: Über das Auftreten und den Umsatz der α-Glycerinphosphorsäure bei der enzymatischen Kohlenhydratspaltung, *Biochem. Zeit.*, **264**: 40 (1933).
——— und ———: Über die phosphoryliertens Zwischen-produkte und die letzten Phasen der alkoholischen Gärung, *Biochem. Zeit.*, **267**: 313–348 (1933).
——— und ———: Über die Isolierung der isomeren Phosphoglycerinsäuren (Glycerinsäure-2-Phosphorsäure und Glycerinsäure-3-Phosphorsäure) aus Gäransätzen und ihr enzymatisches Gleichgewicht, *Biochem. Zeit.*, **276**: 239 (1935).
——— und ———: Über die enzymatische Umwandlung von Glycerinaldehydphosphosäure in Dioxyacetonphosphorsäure, *Biochem. Zeit.*, **279**: 40 (1935).
——— und ———: Über die enzymatische Umsatz der synthetischen Phosphobrenztraubensäure (enol-Brenztraubensäure-phosphorsäure), $CH_2{=}C(OH_2PO_3)$-COOH, *Biochem. Zeit.*, **280**: 99 (1935).
——— und ———: Die Umesterungsreaktion der Phosphobrenztraubensäure bei der alkoholischen Zuckergärung, *Biochem. Zeit.*, **281**: 249 (1935).
——— und ———: Über den Hauptweg der Milchsäurebildung in der Muskulatur, *Biochem. Zeit.*, **283**: 83 (1935).
——— und K. Lohmann: Über den Nachweis von Triosephosphorsäure als Zwischenprodukt bei der enzymatischen Kohlenhydratspaltung, *Naturwissenschaften*, **22**: 134 (1934).
——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. *Naturwissenschaften*, **22**: 220 (1934).
——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure, *Biochem. Zeit.*, **271**: 89 (1934).
——— und ———: Über die enzymatische Umwandlung von Phosphoglycerinsäure in Brenztraubensäure und Phosphorsäure, *Biochem. Zeit.*, **273**: 60 (1934).
——— und ———: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsaure und Dioxyacetonphosphorsäure. II. Die negative Wärmetönung der enzymatischen Spaltung, *Biochem. Zeit.*, **273**: 73 (1934).
——— und ———: Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. III. Über Abfangen der Triosephosphorsäure mit Bisulfit und die Verbreitung des Ferments "Zymohexase" in den verschiedenen Zellarten, *Biochem. Zeit.*, **273**: 413 (1934).
——— und ———: Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. IV. (Berichtigung zur II. Mitteilung), *Biochem. Zeit.*, **275**: 430 (1935).
———, ———, und P. Schuster: Über die Aldolase ein Kohlenstoffverknüpfendes Ferment. Aldolkondensation von Dioxyacetonphosphorsäure mit Acetaldehyd. *Biochem. Zeit.*, **286**: 301, 319 (1936).
——— and P. Oesper: The Mechanism of the Oxidative Reaction in Fermentation, *Jour. Biol. Chem.*, **170** (No. 1): 1–22 (1947).
———, P. Ohlmeyer, W. Gentner, und H. Meir-Leibnitz: Studium der Zwischenreaktionen der Glykolyse mit Hilfe von radioaktivem Phosphor, *Biochem. Zeit.*, **298**: 396 (1938).
———, ———, und W. Möhle: Über die Koppelung zwischen Oxydoreduktion und Phosphatveresterung bei der anaeroben Kohlenhydratspaltung, *Biochem. Zeit.*, **297**: 90 (1938).

——— und W. Schulz: Eine neue Bestimmungsmethode der Phosphoglycerinsäure, *Biochem. Zeit.*, **297**: 60: 1938.

Michaelis, L.: Chemistry of Alcoholic Fermentation, *Ind. Eng. Chem.*, **27**: 1037 (1935).

———, V. Moraques-Gonzales, and C. V. Smythe: Action of various dyestuffs on fermentation and phosphate synthesis in yeast extract, *Enzymologia*, **3**: 242 (1937).

——— and C. V. Smythe: Influence of Certain Dyestuffs on Fermentation and Respiration of Yeast Extract, *Jour. Biol. Chem.*, **113**: 717 (1936).

Myrbäck, K.: Die Co-Zymase und ihre Bestimmung, *Zeit. physiol. Chem.*, **177**: 158 (1928).

———: Co-Zymase, *Ergeb. Enzymforsch.*, **2**: 139 (1933).

———: Zur Kenntnis der Co-Zymase, V, *Zeit. physiol. Chem.*, **233**: 95 (1935).

——— und H. von Euler: Gärungs-Co-Enzym (Co-Zymase) der Hefe, IV, *Zeit. physiol. Chem.*, **138**: 1 (1924).

——— und ———: Co-Zymase und die Aktivierung der Gärung frischer Hefe durch Hefenextrakten, *Zeit. physiol. Chem.*, **176**: 258 (1928).

——— und B. Örtenblad: Zur Kenntnis der Co-Zymase, IV, *Zeit. physiol. Chem.*, **233**: 87 (1935).

Neuberg, C.: Development of Chemistry of Fermentation, *Am. Brewer*, **75**: 22–30 (May, 1942).

——— und A. Hildesheimer: Über zuckerfreie Hefegärungen, I, *Biochem. Zeit.*, **31**: 170 (1911).

——— und J. Hirsch: Über den Verlauf der alkoholischen-Gärung bei alkalischer Reaktion. II. Gärung mit lebender Hefe in alkalischen Lösungen, *Biochem. Zeit.*, **96**: 175 (1919).

——— und ———: Wirkungsweise der Abfangmethode bei der Acetaldehyd-Glycerin-Spaltung des Zuckers, *Biochem. Zeit.*, **98**: 141 (1919).

——— und M. Kobel: Die desmolytisches Bildung von Methylglyoxal durch Hefen-,enzym, *Biochem. Zeit.*, **203**: 463 (1928).

——— und ———: Die Bildung von Brenztraubensäure als Durchgangsglied bei der alkoholischen Zückerspaltung. Ihre Isolierung als Hauptprodukt der Gärung, *Biochem. Zeit.*, **216**: 493 (1929).

——— und E. Reinfurth: Die Festlegung der Aldehydstufe bei der alkoholischen Gärung. Ein experimenteller Beweis der Acetaldehyd-Brenztraubensäure-Theorie, *Biochem. Zeit.*, **89**: 365 (1918).

Nilsson, R.: Einige Betrachtungen über den glykolytischen Kohlenhydratabbau, *Biochem. Zeit.*, **258**: 198 (1933).

——— und H. von Euler: Co-Zymase und Adenosintriphosphat, *Zeit. physiol. Chem.*, **204**: 204 (1932).

Oulmeyer, P.: Über die Gargeschwindigkeit der Hexosediphosphorsäure und ihre Umesterung mit Adenylsäure, *Biochem. Zeit.*, **283**: 114–127 (1935).

———: Darstellung von reiner Dihydrocozymase in Substanz, *Biochem. Zeit.*, **297**: 66 (1938).

Ostern, P., und A. J. Guthke: Über die Darstellung der Phosphoglycerinsäure, *Zeit. physiol. Chem.*, **248**: 155 (1937).

Parnas, J. K.: "Handbuch der Enzymologie," II, Leipzig, 1940.

———, C. Lutwak-Mann, und T. Mann: Über die Verkeltung der chemischen Umsetzungen in der alkoholischen Gärung, *Biochem. Zeit.*, **281**: 168 (1935).

Robison, R.: A New Phosphoric Ester Produced by the Action of Yeast Juice on Hexoses, *Biochem. Jour.*, **16**: 809–824 (1922).

——— and E. J. King: Hexosemonophosphoric Esters, *Biochem. Jour.*, **25**: 323 (1931).

Smythe, C. V.: Phosphoric Acid Esters from Yeast Extract, *Jour. Biol. Chem.*, **117**: 135 (1937).

———: An Improved Method of Preparing Hexosemonophosphate from Yeast Extract, *Jour. Biol. Chem.*, **118**: 619 (1937).

Stephenson, M.: "Bacterial Metabolism," 2d ed., Longmans, Green & Company, New York, 1939.

Warburg, O., und W. Christian: Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Zeit.*, **254**: 438 (1932).

CHAPTER VI

BREWING

Brewing, or the production of malt beverages, is the name given to the combined processes of preparing beverages from infusions of grains that have undergone sprouting (malting), and the fermenting of the sugary solution by yeast, whereby a portion of the carbohydrate is changed to alcohol and carbon dioxide. It is an ancient industry and was probably invented by the Egyptians.

The term "malt beverage," according to the Federal Alcohol Administration Act[1] of May, 1937,

> . . . means a beverage made by the alcoholic fermentation of an infusion or decoction, or combination of both, in potable brewing water, of malted barley with hops, or their parts, or their products, and with or without other malted cereals, and with or without the addition of unmalted or prepared cereals, other carbohydrates or products prepared therefrom, and with or without the addition of carbon dioxide, and with or without other wholesome products suitable for human food consumption.

Beer, ale, porter, and stout are examples of malt beverages.

Composition of Beers.—The substances found in a beer will depend largely upon the nature and quality of the raw materials, the treatment of the sprouted grain or malt used in mashing, and the character of the ensuing fermentation, but storage and finishing operations will likewise affect the final composition.

In a normal beer one may expect to find carbohydrates—such as dextrin, maltose, and glucose—and protein derivatives such as peptones, amino acids, and amides, these products arising mainly as the result of the action of the enzymes of the malt. Hops contribute bitter substances, resins, essential oil, and tannic acid, but a portion of some of these substances is lost during the subsequent brewing operations. As a result of the alcoholic fermentation, the sugars of the wort are converted, in part at least, to ethanol and carbon dioxide, with much smaller quantities of glycerol and acetic acid; while some of the amino acids are transformed to higher alcohols and acids, for example, succinic acid. Lactic acid may be added to the wort or may accumulate as the result of

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Federal Administration Act as in effect on May 15, 1937.

the action of lactic acid bacteria. Salts are always found and usually traces of oil. The finished beer contains 85 to 92 per cent of water by volume. Thus a beer is not a simple beverage but one that is capable of wide variation in composition unless the conditions of brewing are very carefully regulated.

Table 35 gives the analyses of several types of beer.

TABLE 35.—ANALYSES OF SOME MALT BEVERAGES[1]

Variety	No. of analyses	Specific gravity	Water	Carbonic acid	Alcohol by weight	Extract	Nitrogenous substances	Sugar as maltose	Gum and dextrin	Acid as lactic	Glycerin	Ash	Phosphoric acid
Schenk	205	1.0114	91.11	0.197	3.36	5.34	0.74	0.95	3.11	0.156	0.12	0.204	0.055
Lager	258	1.0162	90.08	0.196	3.93	5.79	0.71	0.88	3.73	0.151	0.165	0.228	0.077
Export beer	109	1.0176	89.01	0.209	4.40	6.38	0.74	1.20	3.47	0.161	0.154	0.247	0.074
Bock	84	1.0213	87.87	0.234	4.69	7.21	0.73	1.81	3.97	0.165	0.176	0.263	0.089
Weiss bier	26	1.0137	91.63	0.297	2.73	5.34	0.58	1.62	2.42	0.392	0.092	0.149	0.034
Porter	40	1.0191	88.49	0.215	4.70	6.59	0.65	2.62	3.08	0.281	.....	0.363	0.093
Ale	38	1.0141	89.42	0.201	4.75	5.65	0.61	1.07	1.81	0.278	.....	0.31	0.086

[1] Reprinted by permission, from "Food Inspection and Analysis," 4th ed. by Leach and Winton, published by John Wiley & Sons, Inc., New York.

Extent of Manufacture.—Some idea of the magnitude of the industry may be gained by noting the following table, which gives the malt beverage production for the six leading states in the United States for the Government fiscal years 1936 and 1946. For each state, the number of breweries operated is also indicated.

During the fiscal year 1946, Missouri, New Jersey, and California, in the order named, followed the lead of the first five states listed in Table 36 and produced more than Michigan.

TABLE 36.—MALT-BEVERAGE PRODUCTION, FISCAL YEARS 1936 AND 1946[1]

State	Production in barrels[2]		Breweries operated	
	1936	1946	1936	1946
New York	8,598,081	13,464,043	70	44
Pennsylvania	6,010,171	9,558,788	112	63
Wisconsin	5,736,534	9,054,093	91	61
Illinois	3,578,180	5,968,439	61	39
Ohio	3,432,921	5,917,205	54	39
Michigan	3,249,355	4,126,082	52	24
Total, United States	51,812,062	84,977,700	732*	471

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1936 and 1946.
[2] The standard beer barrel holds 31 gal.
* Number operated during any part of the year.

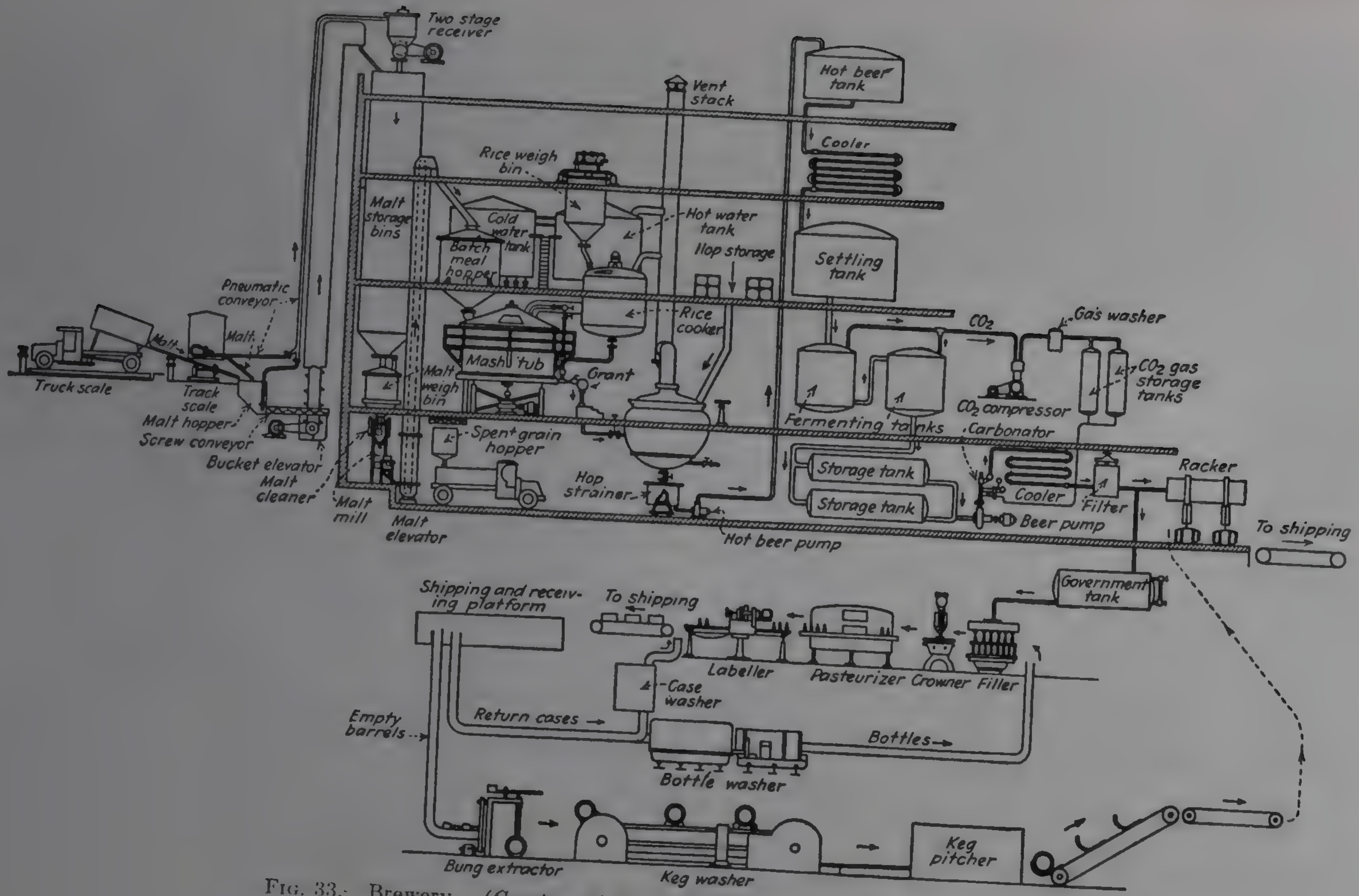


FIG. 33.- Brewery. (*Courtesy of Dr. L. V. Burton, Editor, Food Industries.*)

American Brewing Practice.—Beer produced in the United States is distinctive for two principal reasons. First, the American public prefers a sparkling clear, light, and very pale beer—one that will remain brilliant when very cold or warm. Second, owing to the differences in the composition and properties of our malts and cereals, departure from European procedures has become necessary.

The Brewing Process.—The essential steps in the manufacture of beer include the preparation of malt, mashing, boiling the wort with hops, fermentation, and finishing operations. Malt is prepared by soaking selected barley in water, permitting it to germinate and drying it under carefully regulated conditions. Malting is sometimes carried on as a separate industry, but it is so closely associated with the brewing industry that it may be regarded as an essential part of the whole brewing process. In mashing, ground malt is mixed with heated water and prepared malt adjuncts are usually added. The enzymes liberated by the malt digest the starches, proteins, and some of the other substances present in the mash. The soluble products are dissolved in the water of the mash and form the sweet wort. The mash is filtered, and the resultant clear wort is boiled with hops, strained, cooled, and pitched (inoculated) with selected yeast. A fermentation ensues, which is carried out at a low temperature. For lager beer the temperature is slightly lower than for ale. Following this fermentation, the beer is stored to mature or age. During the finishing operations, the beer is carbonated, cooled, racked, and pasteurized.

Although the principles employed in the production of beer and ale are essentially similar there are minor differences that are carefully regulated. A different treatment of malt is required both as to the length of the germinating period and the kilning of the malt; a higher temperature of fermentation is used, and a different type of yeast. Beer yeasts are so-called "bottom" yeasts, since the fermentation proceeds vigorously in the depths of the tanks and there is a great deposition of cells, while ale is fermented by "top" yeasts that produce great masses of cells in the foam that forms abundantly at the surface. Beer contains larger amounts of unfermented carbohydrates, especially dextrins, while ale has a higher percentage of alcohol. This is in part due to the more complete conversion of the starch to fermentable sugar in the malt used.

Malt.—The preparation of malt is described in Chap. III.

Malt Adjuncts.—In some countries, beer is prepared from malt, hops, and water only, but in the United States malt adjuncts are employed in addition, owing to the fact that the barleys used for the preparation of malt in this country are richer in protein than the barleys used in Euro-

pean countries. A high nitrogen content is usually undesirable for it tends to produce a satiating and relatively unstable beer.

Besides reducing the nitrogen content of the wort, malt adjuncts supply additional carbohydrate to be acted on by the excess of diastase present and to be fermented by the yeast, and they help to produce a beer that is less satiating, paler in color, and relatively more stable.

Malt adjuncts include certain grains and grain products (cooked or uncooked), sugar and sirups, and miscellaneous other carbohydrate products. In normal times in this country, corn and corn products (corn grits, corn flakes, corn sugar), rice, sugar, and sirups are used as the principal malt adjuncts. With a shortage in malt or the common malt adjuncts other materials may be used as is shown in Tables 37 and 38.

TABLE 37.—KINDS AND QUANTITIES OF MATERIALS USED IN THE PRODUCTION OF FERMENTED MALT LIQUORS BY PRINCIPAL STATES DURING THE FISCAL YEAR 1947[1,2]

State	Malt	Corn and corn products	Rice	Wheat	Barley	Sorghum grain
New York	384,823,535	118,587,540	14,850,300	290,890	2,465,320	10,052,225
Pennsylvania	265,300,989	65,454,118	3,031,090	80,200	4,697,577	14,791,383
Wisconsin	271,524,459	100,463,311	27,569,800	.....	664,078	6,753,880
Missouri	187,334,730	21,634,475	61,942,351	.....	250,900	2,547,700
Ohio	178,291,337	58,796,892	13,717,799	15,800	1,474,421	1,800,321
New Jersey	180,033,150	53,989,631	586,520	.....	615,695	5,821,990
California	149,418,577	40,326,641	4,176,450	.....	.....	8,962,179
Total, United States.	2,513,788,652	719,300,506	172,199,735	769,790	13,777,421	88,995,670

State	Soybeans and soybean products	Sugar and sirups	Hops and hop extracts	Cassava and cassava products	Potatoes and potato products	Other materials
New York	737,538	35,298,897	6,474,359	19,574,237	3,160,465	10,500
Pennsylvania	156,812	41,322,489	4,338,023	5,449,939	375,800	
Wisconsin	967,913	12,559,790	4,030,021	24,437,885	218,309	
Missouri	78,112	3,305,265	3,547,782	165,756	394	
Ohio	237,944	15,941,864	2,706,479	2,795,821	1,100	
New Jersey	156,820	16,390,998	2,745,066	2,985,607	175,100	
California	669,072	3,958,878	2,317,918	3,359,423	155,760	
Total, United States.	4,885,118	218,598,828	40,506,913	107,629,982	6,651,977	70,713

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, June 30, 1947.
[2] Quantities in pounds.

A mixture of 20 to 35 per cent of malt adjuncts and 65 to 80 per cent of malt is commonly used in the manufacture of beers in this country.[1]

[1] POZEN, M. A., *Ind. Eng. Chem.*, **26**: 1127 (1934).

In Table 37 are shown the kinds and quantities of materials used in the production of fermented malt liquors.

The quantities of materials used in the production of each barrel of fermented malt liquors are shown in Table 38. These are, of course, average figures based on nationwide production and are intended to convey only a broad picture of the malt-beverage industry. The materials used vary from plant to plant.

TABLE 38.—AVERAGE QUANTITIES OF MATERIALS USED IN THE PRODUCTION OF A BARREL OF FERMENTED MALT LIQUORS[1,2]

Material	Pounds per 31-gal. barrel			Percentage of total material		
	1937	1946	1947	1937	1946	1947
Malt	37.05	25.457	28.600	71.20	56.40	64.669
Corn and corn products	7.20	9.304	8.180	13.85	20.62	18.500
Rice	3.92	2.834	1.960	7.55	6.28	4.430
Wheat	.....	0.565	0.0088	.....	1.25	0.0199
Barley	.....	1.878	0.157	.....	4.16	0.3549
Sorghum grain	.....	1.842	1.012	.....	4.08	2.292
Soybeans and soybean products	.....	0.079	0.0556	.....	0.18	0.126
Sugar and sirups	3.22	2.664	2.490	6.19	5.90	5.630
Hops and hop extracts	0.63	0.442	0.461	1.21	0.98	1.042
Cassava and cassava products	.....	.....	1.225	.....	.....	2.770
Potatoes and potato products	.....	.....	0.0757	.....	.....	0.171
Other materials	.....	0.065	0.0008	.....	0.15	0.0018
Total material per barrel	52.02	45.130	44.226	100.00	100.00	100.00
Total malt adjuncts[3]	14.34	19.231	15.165	27.59	42.62	34.289

[1] Calculated from statistics presented in the annual reports of the Commissioner of Internal Revenue, *U.S.. Treas. Dept.*, June 30, 1937; June 30, 1946; and June 30, 1947.

[2] Data are for fiscal years ending June 30.

[3] Total materials less malt, hops, and hop extracts.

Mashing.—The purpose of mashing is to digest and to dissolve as much as possible of the valuable portions of the malt or malt adjuncts.

The sweet wort that results from this enzyme process contains dextrins, maltose, other sugars, pentosans, protein degradation products, minerals, tannin, coloring matter, and other substances.

Mashing Methods.—There are two main methods for mashing: the infusion and decoction methods. Many modifications of these methods are used in practice.

THE INFUSION METHOD.—There are two infusion processes: one of these is the upward method; the other, the downward method. In the upward process, the malt is mixed (doughed in) with water at a tem-

perature of 38 to 50°C. This main mash is permitted to rest (protein rest period) for about 1 hr. at this temperature to favor the action of the proteolytic enzymes. The temperature is then raised to 65 to 70°C. by the doughing in of the cooked starchy malt adjuncts, which are at the boiling temperature. The mash is permitted to stand at this temperature for a few minutes for starch saccharification. The temperature is then brought to about 75°C., or a little above, for the destruction of the enzymes. The mash is filtered at this temperature.

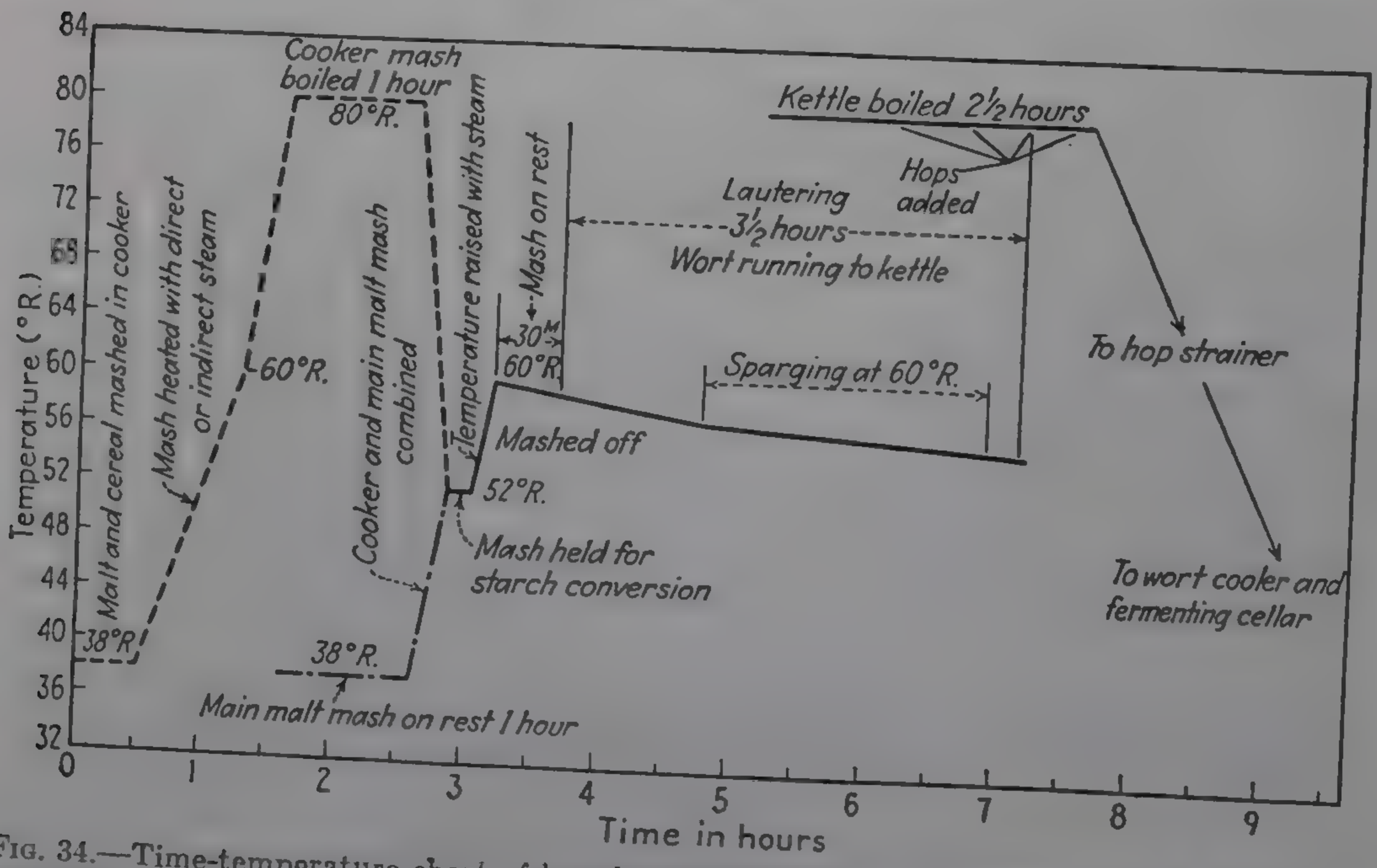


FIG. 34.—Time-temperature chart of brewhouse operations. [*Courtesy of Dr. R. Schwarz, Ind. Eng. Chem.*, **27**: 1031 (1935).]

In the downward infusion process, the initial temperature of the mash water may be about 77°C. The added malt aids in cooling the water to approximately 70°C. A temperature of 65 to 70°C. is maintained, as in the upward infusion process, to permit saccharification. The final temperature of the mash is lower than the initial temperature. The downward infusion process is an English method.

THE DECOCTION METHOD.—In this method, the mash is mixed at a lower temperature, occasionally around 40°C., than is the case with infusion processes. The temperature of the mash is raised by steps until a final temperature of about 75°C. has been obtained.

A portion of the initial mash, approximately one-third, is withdrawn, heated, and boiled for a short period of time and then returned to the main mash. The heated portion raises the temperature of this entire mash. The enzymes in the boiled portion have been destroyed, but the

cell walls of the grain are softened and the starch liquefied. Diastatic action is thus facilitated by this process.

The mash may be maintained for a while at the new temperature. Thereafter another portion of the mash may be removed, boiled, and returned to the main mash. The process may be called a one-, two-, three-mash process or single-, double-, or triple-mashing process, depend-

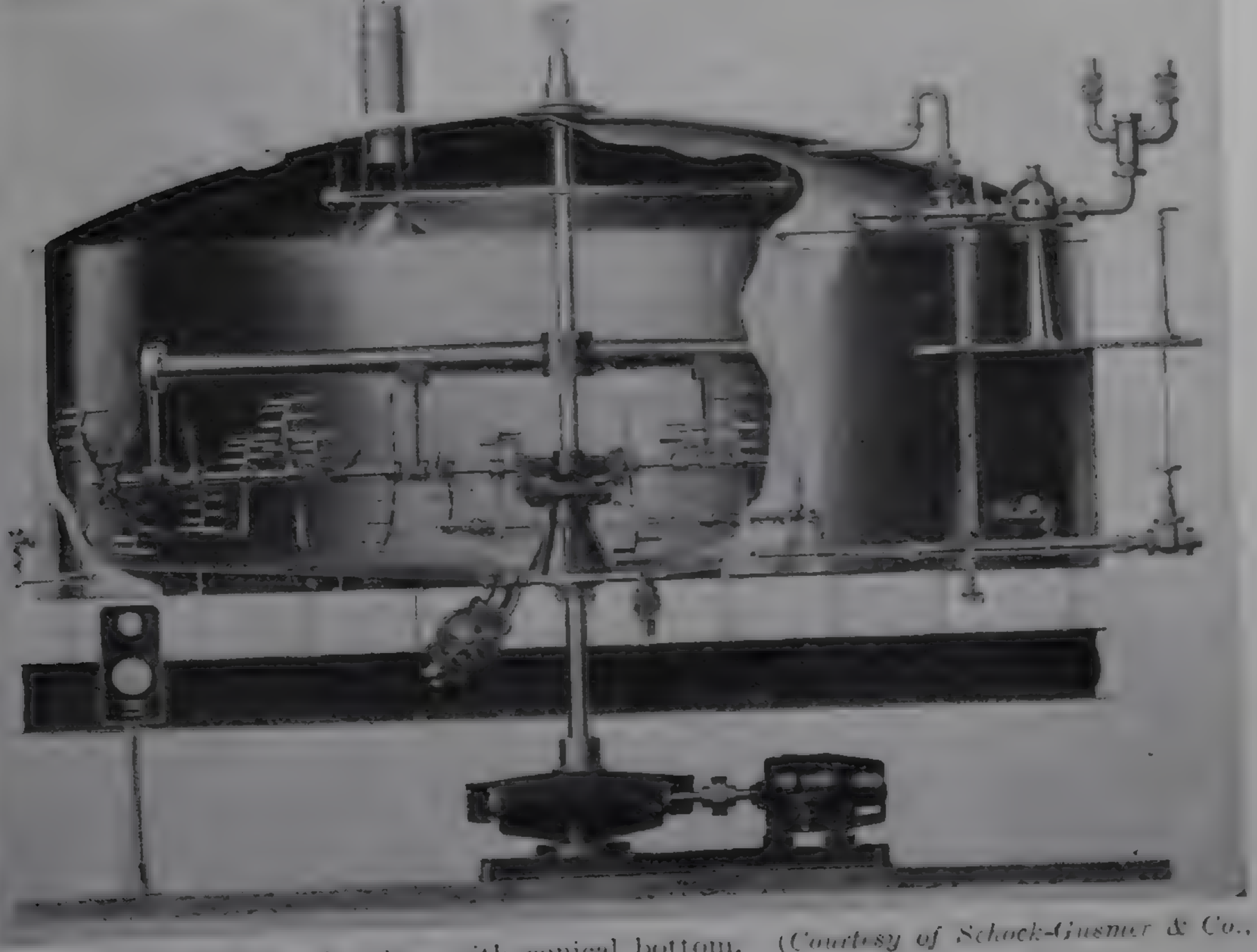


FIG. 35.—Vertical mash mixer with conical bottom. (*Courtesy of Schoch-Gusmer & Co., Inc., Hoboken, N. J.*)

ing on the number of portions that have been heated and returned to the mash.

In a three-mash process, according to Hopkins and Krause, the mash may be held at the initial temperature (40°C.) in order to extract the enzymes and favor proteolysis and the action of phytase. The mash may then be held at 50°C. for "full proteolysis and peptonization"; at 60 to 65°C. for saccharification; and at 70 to 75°C. for dextrinization of any unconverted starch by α-amylase and complete extraction.

Comparison of Methods.—The decoction process usually yields a higher percentage of extract than the infusion process. But the flavor of the wort obtained from the infusion process may be better than that of the

decoction process, because bitter resins are not dissolved to such an extent. Either method should produce satisfactory results, however.

Important Considerations in Mashing.—In mashing, the proper preparation of the malt and uncooked malt adjuncts; the treatment of the mash water, if necessary; and the control of temperature, pH, mash concentration, and time are important.

Preparation of Malt.—Malt is selected, cleaned, and ground. Grinding, to be satisfactory, must be accomplished according to the variety of malt and the type of equipment being used. There should be proper distribution of the ground malt into husks, coarse grits, fine grits and flour. The presence of unground kernels results in a loss of extract, while the production of too finely pulverized malt interferes with the filtration of the wort. The properly prepared malt is mixed with a measured quantity of water at the correct temperature in the mash kettle.

Treatment of Malt Adjuncts.—Unmalted cereals, such as corn meal, corn grits, rice, or wheat are mixed with water and a small amount of malt, agitated and brought gradually to a boiling temperature. The mash, known as a converter mash, is then cooked with or without the use of pressure to gelatinize the starch and render it soluble. The converter mash is eventually added to the main mash containing the malt.

Malt adjuncts that exist in the prepared state, such as cereal flakes, are added directly to the main mash without any preliminary cooking.

Sugars and sirups are usually added to the wort.

Composition of Mash Water.—The composition of the water that is to be used in brewing is very important. The quantity and character of the dissolved salts are of much significance while pH is of secondary interest. Water containing calcium and magnesium sulphates with a permanent hardness of between 200 to 300 parts per million (expressed as calcium carbonate)[1] is usually suitable. On the other hand, soft water is required for the brewing of certain light lager beers. Filtration, clarification, and stability are influenced to some extent by the type of water used. "Burtonizing" is the name given to a process of artificially increasing the permanent hardness of water for ale and beer-making. It is so called from Burton-on-Trent in England where excellent ales have long been produced and the water supply is regarded as practically perfect for this purpose. (For a further discussion of this subject, the reader is referred to "Biochemistry Applied to Malting and Brewing," by Hopkins and Krause.)

Temperature.—The temperature of mashing is a very important factor in determining the type of beer to be produced. A high temperature is more favorable to the action of α-amylase than of β-amylase. Hence

[1] SCHWARZ, R., Brewing Processes, *Ind. Eng. Chem.*, **27**: 1031 (1935).

a higher proportion of dextrin will be produced when the mash is converted at a relatively high temperature. The fermentation of a wort containing a relatively large proportion of dextrin results in a low-alcohol beer. On the other hand, the use of a low conversion temperature favors the production of a larger proportion of sugar and less dextrin and, subsequently, a high-alcohol beer, or ale.

When the action of α-amylase is favored and that of β-amylase is inhibited, dextrins will accumulate at the expense of maltose. Dextrins are colloidal and, when present in a wort in sufficiently high concentration, will cause a slow filtration of the wort. Sparging will likewise be difficult, and considerable extract will be left in the spent grains. Furthermore, dextrins are not fermented by beer yeasts. It is this fact that permits one to control the alcohol in a given beer more readily.

The following table illustrates the effect of the temperature of conversion on the ratio of sugars to dextrins:

TABLE 39.—EFFECT OF THE TEMPERATURE OF CONVERSION ON THE RATIO OF SUGARS TO DEXTRINS[1]

Conversion Temperature, Degrees Centigrade	Ratio of Sugar to Nonsugar
64	1:0.37
66	1:0.40
68	1:0.48
70	1:0.52
72	1:0.57

[1] POZEN, M. A., Enzymes in Brewing, *Ind. Eng. Chem.*, **26**: 1127 (1934).

By carefully controlling the temperature, it is thus possible to regulate the relative proportions of sugar to dextrins.

Starch liquefaction is most rapid at 70 to 75°C., while saccharification proceeds most rapidly at approximately 65°C. The optimum temperature for a given conversion is governed by the pH of the mash, its concentration, and other factors.

Temperature very definitely affects the type of proteolysis that occurs during mashing. At a conversion temperature of about 60°C., nitrogenous compounds of high molecular weight are formed. These compounds are valuable in the production of palatefulness, foam, and foam stabilization in beer. A greater proportion of amino nitrogen is formed at 50°C. The derivatives of low molecular weight, the amino acids in particular, are useful as food for the yeast in subsequent fermentation. An undue amount of proteolysis, especially the production of amino nitrogen in quantities greatly in excess of those required for the nutrition of the yeast, should be avoided.

pH.—By properly adjusting the pH of the mash, the quantity of extract will be increased, while subsequent clarification and filtration will be facilitated. The pH of the mash will influence the activity of the enzymes, each of which functions best at a given pH, which is dependent also on the temperature, concentration and nature of the mash, and other factors.

According to Hopkins and Krause, the yield of extract in a mash is maximum at a pH of around 5 to 5.2. β-Amylase is very active at this pH as well as protease. Maltose formation and attenuation are best at a pH of approximately 5.5. This value is excellent for filtration also. At pH values below 5, amino nitrogen is formed at a maximum rate.

Tannin and bitter resins are dissolved from the husk more readily at higher pH values, since they are weakly acidic in nature. Color, likewise, is extracted better at higher pH values.

During mashing, the pH varies, but it may be in the neighborhood of 5.8 at the beginning of the process. Lager beer, when ready for cooling, may indicate a pH of about 5.2 to 5.5.

High-grade lactic, sulphuric, phosphoric, or other acids may be used to adjust the reaction of the medium. It is essential, however, to use an acid that will have no deleterious effect on the flavor of the beer or its value to the consumer.

Concentration of Mash.—Since mashing is an enzymic process, the concentration of the mash influences the quality and quantity of the extract.

Time.—In order to control the composition of the final mash, it is important to regulate the time permitted for mashing. The time allowed will be intimately connected with the other factors of enzyme reaction—temperature, pH, concentration of substrate and menstruum.

During the last part of mashing especially the grains and coagulated proteins will settle to the bottom of the mashing tub. Although some manufacturers permit settling to take place in this tub and filter the wort by allowing it to flow down through the layer of grains and coagulated proteins, the wort passing out through a false bottom, others convey the material from the mash tub to a lauter tub. The lauter tub is a special filtering tank with a false bottom. Those which are wide and shallow give the quickest and best results. The filtered wort should be clear.

The grains that remain after the wort has been drawn off are "spent." They are extracted or leached several times with water at a temperature of 75 to 80°C., preferably 75°C., the process being known as "sparging." The sparged grain should finally contain less than 1 per cent of residual soluble extract. It is used as cattle food, either in wet form or after drying.

The wort and spargings are placed in a brew kettle, usually made of copper, and the hops are added in the proportion of 0.55 to 0.9 lb. per standard beer barrel of 31 gal.

Boiling the Mash.—Wort containing hops is boiled for several reasons: to concentrate it, to sterilize it, to inactivate the enzymes, to extract soluble substances from the hops, to precipitate coagulated proteins and other substances, and to slightly caramelize the sugar. The addition of the spargings to the main wort dilutes it, making concentration desirable. The danger of the growth of undesirable microorganisms in the wort is lessened by sterilizing it and handling it under aseptic conditions. Inactivation of the enzymes aids in maintaining a wort of fairly constant composition for fermentation.

The substances extracted from hops include bitter acids and resins, essential oil, and tannin. The bitter acids and resins contribute to the palatefulness, colloidal stability, and head retention of the beer. The bitter acids are humulon or α-bitter acid ($C_{21}H_{30}O_5$), and lupulon or β-acid ($C_{26}H_{38}O_4$), respectively. By oxidation and polymerization the acids may be converted to soft resins. Both the acids and their corresponding resins possess antiseptic properties and impart characteristic flavors to beer. Humulon, however, possesses the strongest bitter flavor and the greatest antiseptic action. A third resin, of little value to the brewing industry, is the hard gamma resin.

A large proportion of the antiseptic action of hops is lost during the drying of the hops, their storage before use, boiling them with the wort, cooling, fermentation, and storage of the beer in barrels.[1]

Some flavor is imparted to beer from the essential oil of hops. Since the essential oil is volatile in steam, most of it is lost during the boiling of the wort, unless the hops are added toward the end of the boiling process.

Tannins are extracted principally from the hops during boiling, but some are extracted from the malt. The tannins from barley possess an unpleasant taste. Hence their removal by reaction with the proteins of the wort, before the hops are added, is advantageous.

Tannin aids in the precipitation of some of the nitrogenous substances of the wort. Some of the tannin complexes precipitate out during boiling, but others[2] tend to become insoluble in the cold, giving rise to a chill haze, unless removed during subsequent cooling.

[1] Walker, T. K., A Review of Ten Years' Research on the Antiseptic Constituents of Hops, *Jour. Inst. Brewing*, **38**: 198 (1932).

[2] Luers, H., and C. Enders, Acidity and Protein Turbidities in Beer, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **22**: 329 (1938).

Tannins are negatively charged. They react readily with positively charged proteins, becoming compounds that become less soluble as the temperature decreases. Tannins react less readily with electrically neutral proteins, *i.e.*, proteins that are unstable.

Finding materials some of the tannin of the hops into phlobaphene. Protein-phlobaphene forms also during the boiling of the wort, and, being insoluble in the hot wort, is precipitated out. Oxygen accelerates protein-phlobaphene formation.[4]

Agitation and circulation of the wort during boiling increases the amount of precipitation and hence the quantity of sludge formed. During cooling agitation is also of much advantage in increasing the amount of precipitation. The greater the quantity of unstable compounds removed during boiling and cooling, the less likelihood there is of the formation of precipitates in the finished product.

After the wort has been boiled, it is filtered through a hop strainer to remove the hops and precipitated proteins. The wort may, or may not, be passed into a tank located above the cooler where settling may be permitted for 0.5 to 1 hr. Some cooling takes place in this tank. The wort is then cooled by passing it over or through coolers, preferably with considerable agitation.

During cooling, the wort becomes acidified and the pH* may increase from 4.2 or 4.3 to 5.0 or 5.1.[5] Protein-tannin compounds precipitate out owing to their insolubility at the lower temperatures. A secondary precipitation is induced whereby proteins and hop resins are adsorbed on the surfaces of the protein-tannin compounds.

The rate at which the wort is cooled between the temperatures of 60 and 21°C has a direct bearing on protein-tannin precipitation.[3] Coolers must be designed through which rapid cooling takes

Preventing contamination of the wort during cooling is essential.

Fermentation. Yeasts. Strains of *Saccharomyces cerevisiae* are commonly used in the manufacture of beer. The selection of an appropriate strain is a most important factor in determining the character of the beer.

* pH is a term which represents the negative logarithm of the hydrogen ion pressure in equilibrium with the solution and which is being studied.

[3] Hough, J. S., Processes Which Take Place During Brewing, *Food Technol.*, 10: 74 (1956).

[4] Briggs, D. E., Brewing Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1957.

[5] Hough, J. S., Recent Advances in Brewing Technology, *Food Research*, 3: 209 (1958).

[6] Hough, J. S., "Biochemical Principles," 2d ed., Longmans, Green & Company, New York, 1958.

Brewery yeasts may be classified as top-fermenting yeasts, which are used in the manufacture of ale; and bottom-fermenting yeasts, which are used in the production of lager and other beers.

Saccharomyces carlsbergensis Hansen[1] is a bottom yeast, which has been used in the breweries of Copenhagen and elsewhere. The Saaz yeast and *S. monacensis* are typical bottom yeasts also.

Pitching the Wort.—The addition of yeast to a wort is known as "pitching." Only pure cultures should be used for such purposes.

Wort is placed in tanks located in cellars (not necessarily below the surface of the ground), and maintained at a temperature that depends on the type of beer being manufactured. Some worts are stored at 0 to 6°C.; others may be stored at slightly higher temperatures. The wort is pitched and fermentation follows.

Fermentation.—It is the practice in some breweries to pump the fermenting wort to another fermentation tank after the appearance of a ring of white foam (krausen stage) on the surface of the wort. In so doing, precipitated proteins, hop resins, and some yeast cells are left behind. Since carbon dioxide is used to carbonate beers, the use of closed fermentors with arrangements for carbon dioxide collection is now practiced.

During fermentation the temperature rises several degrees. Modern fermentors are provided with cooling equipment to prevent the temperature from rising too high.

As the fermentation proceeds, various substances accumulate on the surface of the medium, including hop resins, yeast cells, and proteinaceous material. This scum may be removed after the fermentation starts to recede, in order to improve the quality of the beer.

Certain very definite changes take place in the wort during fermentation. At a particular point near the end of the fermentation the yeast flocculates and commences to settle. This change is known as a "break" and depends in part upon the pH for initiation. (Not all yeasts break, however.) A large proportion of the fermentable sugars are transformed to ethyl alcohol, carbon dioxide, glycerol, and acetic acid. Higher alcohols and acids are produced from protein and fat derivatives. Esters are formed from organic acids and alcohols. The pH of the mash drops gradually. Insoluble products precipitate out.

The fermentation of a bottom-fermenting yeast, which is usually carried out at 6 to 12°C., is ordinarily completed in 8 to 10 days, while that of a top-fermenting yeast is completed in a shorter time, 5 to 7 days,

[1] GUILLIERMOND, A., "The Yeasts" (translated and revised by F. W. Tanner), John Wiley & Sons, Inc., New York, 1920.

at a somewhat higher temperature, 14 to 23°C.[1] Beer at the end of this stage is known as "green" or "young" beer.

The beer resultant from the fermentation still contains undesirable substances in suspension. The elimination of the suspended materials by clarification and the use of low temperatures, and the production of "bouquet" in the beer are brought about by subsequent processes.

Maturing.—Beer is stored in refrigerated cellars for periods of 2 weeks to several months, depending on the process being used and the type of beer desired. The temperature is maintained at 0°C. During storage, unstable proteins, yeast, resin, and other undesirable substances precipitate from the beer. The harshness of the "green" beer disappears. Esters are produced, and the beer becomes mellow or matured.

Finishing.—The matured beer is carbonated under pressure, using carbon dioxide that is at least 99.5 per cent pure, until a final carbon dioxide content of 0.45 to 0.52 per cent is obtained. Gas is absorbed by the beer, and oxygen is displaced. Exclusion of air in the finished beer is very important. The greater the amount of air displaced from the beer, the better. The dissolved gas adds to the quality of the beer, aids in the production and retention of foam, and helps to preserve the beer.

An alternate process for carbonating beer is known as the "krausening process." The beer after storage is placed in pressure tanks or containers, and approximately 15 per cent of beer in an initial active stage of fermentation (krausen) is added and mixed with it. The carbon dioxide evolved from the fermenting young beer impregnates the entire tank of beer. Valves or bungs, attached to the pressure tanks, enable one to regulate the final content of carbon dioxide in the beer (0.45 to 0.52 per cent). The krausening process usually requires 3 or 4 weeks. Afterward the beer is stored for an additional period of 3 to 8 weeks.[2] The action of the yeast stops after the sugars have been consumed. Clarification then proceeds.

Carbonated beer is cooled, filtered, and racked. By racking is meant the distribution of the filtered beer into bottles, cans, or other containers. During the first few months of the year 1937, approximately 43 per cent of the beer was packaged. Bottled beer is usually pasteurized for 20 min. at a temperature of 60 to 61°C.

Defects in Beer.—*Turbidities* in beer may be caused by a number of factors: unstable protein,[3] protein-tannin complexes, starch, resin, and microorganisms.

Gluten, or albumin, turbidity occurs at a low temperature generally.

[1] POZEN, *loc. cit.*
[2] SCHWARZ, *loc. cit.*
[3] LUERS and ENDERS, *loc. cit.*

This type of turbidity is likely to appear when the malt has been improperly dried in the kiln or when a barley with very high protein content is used. Warming the beer causes the turbidity to disappear.

Oxidation turbidity or haze is due in part to protein-tannin compounds. The presence of oxygen; the shaking of the beer during its transportation; the collisions of beer bottles, which impart supersonic and high-pitched sound vibrations; and sunlight affect the formation of oxidation haze. Saturation of the beer with carbon dioxide does much to prevent this turbidity.

Tannin-protein hazes appear also at low temperatures. In order to produce stable, chillproof beers, which will not become hazy or turbid when cold, the use of a small amount of a proteolytic enzyme preparation is advantageous. The enzymes are usually added after the fermentation although they may be added earlier. The enzymes in an acid medium, such as is found in a beer, render the beer stable and chillproof. Credit is due to Wallerstein[1] for this discovery.

Starch turbidities develop as a result of the improper conversion of starch during mashing. Lack of proper digestion at this time may be due to the use of a malt in which the diastase has been destroyed during the kilning. Sparging with water at a temperature much higher than 80°C. may also result in the production of turbidity. Amylases may be added to the storage vats to remove starch turbidity.

The presence of resin oil containing pitch may, rarely, cause turbidity, as may calcium oxalate. Proper filtration will prevent both types of turbidities.

Yeast turbidity may be due to lack of proper clarification during the secondary fermentation, which in turn is caused by an unsatisfactory wort. The use of chips, or krausening (the addition of beer in an active state of fermentation) is usually effective in correcting this type of turbidity. Wild yeasts, especially of the *Saccharomyces pastorianus* III species, produce turbidity. By excluding air and keeping the concentration of fermentable sugars in the beer low, growth of yeasts is inhibited. A low rH, 12 or below, will also inhibit the growth of yeasts according to De Clerck.[2] The use of pure cultures and proper sanitation of the plant should prevent the access and development of wild yeasts.

Among the turbidities caused by bacteria, those produced by sarcinae are most common, especially in bottom fermentations. Bacterial turbidities are less common than turbidities caused by yeasts and frequently will contain yeasts as well as bacteria. Aseptic technique, the use of

[1] Wallerstein, L., U.S. Patents 995,820 and 995,824, 1911.

[2] De Clerck, *Jour. Inst. Brewing*, **40**: 407 (1934).

pure cultures of yeasts, sanitation of the brewery, and a high content of hop antiseptics should prevent bacterial turbidities.

Faulty beer may be the result of the use of a low-grade raw material in the mash; the use of hops of poor quality, too much hops, or prolonged boiling of the hops; contact of the beer with iron, causing an inky taste; contact with tin; an unsuitable brewing water; young or green beer; or carbon dioxide deficiency; etc.

Beer Infection.—The term "beer infection" or "beer disease" is commonly used in describing the undesirable condition that occasionally exists in beers or ales as the result of the presence of microorganisms, chief among which are the bacteria. The microbial agents causing the symptoms may be designated as beer-infection microorganisms or beer-disease microorganisms.

The infection bacteria described in literature are included in five families: the *Pseudomonadaceae*, *Bacillaceae*, *Bacteriaceae*, *Micrococcaceae*, and *Lactobacteriaceae*. However, the bacteria of only three of these families, *Pseudomonadaceae*, *Bacteriaceae*, and *Lactobacteriaceae*, are of particular significance to the brewing industry. The actual number of genera involved is also small, as illustrated by Tosic,[1] who reviewed the subject of beer-infection organisms in 1945 and who reported that beer infections are caused by species of the following genera: *Acetobacter*, *Lactobacillus*, *Streptococcus*, *Flavobacterium*, and *Achromobacter*. There appears to be some doubt as to whether bacteria of the genera *Bacillus* and *Micrococcus* cause infections of malt beverages. The mere fact that sporeformers exist in the raw materials used in brewing is no indication that they are producers of infection.

At this point it may be well to consider some of the conditions encountered in the brewing operation that have in general pronounced effects on bacteria and that, in fact, may determine whether or not an organism will survive or develop in the beer. These conditions are as follows:

1. The constituents of the wort are usually boiled for about 2.5 hr.
2. The yeast fermentations are carried out at low temperatures.
3. The pH values of the wort and beer are low.
4. Hop antiseptics are present in the wort and beer.
5. The beer is usually pasteurized.
6. Anaerobic conditions usually prevail during fermentation and in the beer.
7. Ethyl alcohol is present in the fermentation mash and in the beer.

It is entirely conceivable that an unusually heat-resistant sporeformer, for example, strain of one of the species of the genera *Bacillus* or *Clos-*

[1] Tosic, J., *Brewers Digest*, **20** (No. 9): 99–103 (1945).

tridium, might survive the long boiling period of 2 to 2.5 hr. to which the wort is subjected.

However, such bacteria are commonly Gram-positive and sensitive to hop antiseptics. A surviving sporeformer in order to cause infection would have to be tolerant of a relatively low pH, of anaerobic conditions, and of the alcohol normally found in a beer or ale. Thus, there are ample reasons why sporeformers may not be associated with beer infections.

Bacteria of the species of the *Acetobacter*, *Achromobacter*, *Flavobacterium*, *Lactobacillus*, and *Streptococcus* are destroyed within a relatively short time at temperatures well below the boiling point. Hence, if beverages contain these organisms, they have generally become infected during one or more of the operations following the boiling of the wort.

The species of the genus *Acetobacter* are rod-shaped, though involution forms may occur, Gram-negative, catalase positive (with the exception of *A. peroxidans*), aerobic, and grow usually in the range of 5 to 8 to 35 to 40°C. These bacteria produce acetic acid aerobically from ethyl alcohol and are tolerant of acid and of hop antiseptics in the amounts commonly found in beers. They may be responsible for the production of acidity or sourness in beers due to acetic acid. Two other species, *A. capsulatum* and *A. viscosum*, may produce ropiness in beer, while a third species, *A. turbidans*, may give rise to turbidity and sourness.[1,2] The latter organism apparently has the ability to develop when only a very small amount of oxygen is present.

The acetic acid bacteria, with the exception noted above, are dependent in their action upon the presence of oxygen. Consequently infections by species of the *Acetobacter* are limited to those cases where the fermented wort or beer is exposed to oxygen, as for example, in cooks stored for a prolonged period, in empty beer barrels, and in the pitching yeast. Control is exercised by excluding oxygen from the fermented product and in using pure cultures of yeasts.

Two genera of the family *Lactobacteriaceae*, namely *Lactobacillus* and *Streptococcus*, contain species which may be causes of serious trouble. Bacteria of the genus *Lactobacillus* are rod-shaped, Gram-positive, catalase-negative, microaerophilic, and tolerant to acid. They may be tolerant to hop antiseptics or often adapt themselves to its presence. *L. pastorianus*,[3,4] synonyms of which are *Bacille des Bières Tournées* Pasteur and *Saccharobacillus pastorianus* Van Laer, gives rise to sourness in beers

[1] COSBIE, A. V. C., J. TOSIC, and T. K. WALKER, *Jour. Inst. Brew*, **47**: 382 (1944); **48**: 82 (1942).

[2] COSBIE, A. V. C., J. TOSIC, and T. K. WALKER, *Jour. Inst. Brew.*, **49**: 88 (1943).

[3] SHIMWELL, J. L., *Wallerstein Labs. Communs*, **4** (No. 11): 41 (1941).

[4] TOSIC, *loc. cit.*

and a silky type of turbidity. It may produce lactic, acetic, and formic acids, alcohol, and carbon dioxide from fermentable sugars and grow in the range of 11 to 37°C.

According to Shimwell,[1] *Lactobacillus pastorianus* is probably the most common defect-producing organism to be found in top fermentation breweries. Although difficult to remove entirely from a brewery, once it has gained entrance, the problem may be attacked in several ways. The plant should be kept clean, the yeast used for pitching should be pure, the residual fermentable extract should be kept at a minimum, the pH should be as low as possible consistent with the production of a quality product, the hop rate should be high at the time of racking, and finally the beer should be pasteurized, because *L. pastorianus* is destroyed by effective heat treatment.

The streptococci causing trouble in beer are associated with such defects as "sarcina sickness," sourness, turbidity, and ropiness. Sarcina sickness is a disease of beer characterized by a honey-like odor. This particular odor is due to the production of diacetyl by the bacteria, which combined with the normal odor of the beer, produces the honey-like aroma. (Refer to the section concerned with butter starters.)

The species of bacteria concerned in the production of sarcina sickness, turbidity, sourness, and ropiness is *Streptococcus damnosus* (Claussen) Shimwell and Kirkpatrick. A synonym of this organism is *Pediococcus damnosus* Claussen. Shimwell[1] also lists as possible synonyms *P. perniciosus* Claussen, *P. cerevisiae* Balcke, and *P. sarcinaeformis* Reichard.

Streptococcus damnosus is nonsporeforming, Gram-positive, catalase-negative, anaerobic, tolerant to acid but with an optimum pH of 5.0 to 6.0.

Two varieties of *Streptococcus damnosus* have been described. These are *S. damnosus* var. *viscosus* and *S. damnosus* var. *pentosaceus* (Walters). The first of these varieties was isolated from Scotch ale by Claussen and is characterized by the production of slime in beers that are saturated with carbon dioxide. In flat beers, sarcina sickness is produced rather than slime.

Streptococcus damnosus var. *pentosaceus* (originally named *S. tetragenus* var. *pentosaceus*) was isolated from Australian beer by Walters.

The family *Bacteriaceae* contains two genera which are of considerable importance to the brewing microbiologists: *Achromobacter* and *Flavobacterium*. *A. anaerobium* produces turbidity and off-odors in beers. *F. proteus* is an infection of brewers' yeast.

Achromobacter anaerobium, a Gram-negative, nonsporeforming anaerobe, is tolerant of acids and hops. Actually it will grow at a pH of less than 4 and is not inhibited by the action of hop antiseptics in the con-

[1] SHIMWELL, *op. cit.*, pp. 41–48.

TABLE 40.—A SUMMARY OF SOME INFORMATION CONCERNING THE DEFECTS OF BEER CAUSED BY BACTERIA[1]

Defect	Specific causative agent	Morphology and size[2] (in microns)	Gram reaction	Catalase reaction	Relation to oxygen	Reaction to acid	Reaction to hops	Relation to temperature	Products
			Family—*Pseudomonadaceae*						
Sourness	*Acetobacter aceti*	0.4–0.8 by 1.0–2.0	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. acetosum	0.4–0.8 by 1.0	Neg.	Pos.	Aerobic	Tolerant	Tolerant	Range, 8–36°C.	Acetic acid
	A. industrium	0.3–0.8 by 2.4–20	Neg.	Pos.	Aerobic	Tolerant	Tolerant	Range, 8–35°C.	Acetic acid
	A. kützingianum	Short, thick rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant	Range, 7–42°C.	Acetic acid
	A. lindneri	Rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. mobile	Rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. oxydans	0.8–1.2 by 2.4–2.7	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. pasteurianum	0.4–0.8 by 1.0	Neg.	Pos.	Aerobic	Tolerant	Tolerant	Range, 5–40°C.	Acetic acid
	A. suboxydans	Short rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. xylinum	Short rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
Ropiness	*A. capsulatum*	Coccoid rods 0.6–1.5	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
	A. viscosum	.....	Neg.	Pos.	Aerobic	Tolerant	Tolerant	.....	Acetic acid
Sourness and turbidity	*A. turbidans*	Short rods at 25°C., involution forms at 40°C.	Neg.	Pos.	Aerobic to microaerophilic	Tolerant	Tolerant	Grows in beers at 13°C., destroyed in 10 min. at 52°C.	Acetic acid
			Family—*Bacteriaceae*						
Turbidity, off-odors, and off-flavors	*Achromobacter anaerobium*	Rods 1–1.5 by 2–10	Neg.	....	Anaerobic	Tolerant	Tolerant	Destroyed in 5 min. or more at 60°C.	

Infection of brewers' yeast	*Flavobacterium proteus*	0.8–1.2 by 1.5–4.0 Highly pleomorphic	Neg.	....	Facultative anaerobe	No growth at pH 4.0 Opt. pH = 5.0	.....	Grows in beers at 18°C., destroyed in 5 min. at 54°C.	
Family—*Lactobacteriaceae*									
Sourness, silky turbidity	*Lactobacillus pastorianus*	0.5–1.0 by 7–35	Pos.	Neg.	Microaerophilic	Tolerant	Tolerant	Range, 11–37°C.	Lactic, acetic, and formic acids; alcohol, CO_2
Sarcina sickness, sourness, turbidity, ropiness	*Streptococcus damnosus*	Cocci in pairs, tetrads, chains, clumps. About 1	Pos.	Neg.	Anaerobic	May reduce pH to 3.8	Sensitive but may acquire resistance	Opt. 21–25°C.	Lactic acid, diacetyl
	S. damnosus var. *viscosus*	Cocci in pairs, tetrads, chains	Pos.	Neg.	Anaerobic	May reduce pH to below 4	Sensitive but may acquire resistance	.....	Diacetyl, acid, etc.
	S. damnosus var. *pentosaceus*	Cocci in pairs, tetrads, chains	Pos.	Neg.	Anaerobic	Grows at pH 3.5	Sensitive but may acquire resistance	Grows at 15°C.	Diacetyl, acid, etc.

[1] Information obtained from articles by Tosic, Shimwell, and others, which are referred to in the references at the end of this chapter.
[2] Data secured from Bergey's "Manual of Determinative Bacteriology" and other sources.

centrations used in breweries. It grows only in "primed" beers but rapidly when the temperature is elevated. Glucose and fructose but not maltose are utilized. A silky turbidity and odors of hydrogen sulphide (slight) and of apples are produced. Ethyl alcohol and carbon dioxide are formed. The organism is destroyed by heat-treatment for 5 min. or longer at 60°C.

The infection of brewers' yeast, caused by *F. proteus*, has been described by Shimwell,[1] and Shimwell and Grimes.[2] Shimwell isolated the organism from "pitching" yeast. It is a Gram-negative, nonspore-forming, facultative anaerobe, which produces ethyl alcohol and acid and gives rise to a parsnip-like odor and flavor in both hopped and unhopped wort. Its activities, however, are restricted to the primary fermentation on account of its inability to grow at a pH of about 4.2. Since the pH of the wort may be 5.2 or more at the beginning of the fermentation, it may grow until the lowered pH inhibits further development.

Table 40 summarizes some information concerning the defects of beer caused by bacteria.

Definitions.

Lager beer is literally stored beer. The term "lager" is derived from "lagern," a German verb meaning "to store." According to this definition, all beer would be lager beer. Lager beer is produced by bottom fermentation and is rather high in alcohol and extract with a relatively low proportion of hops.

Bock beer is a heavy beer, dark in color and high in alcohol. It is brewed for consumption in the early spring.

Ale is produced by top fermentation, is pale in color, tart in taste, high in alcohol and contains more hops than does beer.

Porter is a dark ale, high in extract and sweeter than the usual ale in taste. It is brewed from dark or black malt (malt roasted at a high temperature) to produce a wort of high extract. The flavor of hops is less distinct than that of normal ale.

Stout is a strong porter that is high in alcohol and extract. It is dark in color and possesses a sweet taste and strong flavor of malt. The hop flavor is more pronounced than that of porter.

Weiss bier, a beer made mainly from wheat, is produced by top fermentation. It is rather light, possesses a marked flavor of malt and hop, is tart, and contains a large quantity of natural fermentation gas. It is likely to be turbid in appearance.

[1] SHIMWELL, J. L., *Jour. Inst. Brew.*, **42**: 119 (1936).
[2] SHIMWELL, J. L., and M. J. GRIMES, *Jour. Inst. Brew*, **42**: 348 (1936).

Cereal beverage is beer containing less than 0.5 per cent alcohol. It is sometimes known as "near beer."

Brewery Thermometers.—The brewer frequently uses the Reaumur thermometer instead of the Fahrenheit or Celsius (centigrade) scales. A comparison of a few points on the three scales is given in the following table:

TABLE 41.—A COMPARISON OF SOME POINTS ON THE REAUMUR, CENTIGRADE, AND FAHRENHEIT SCALES

Reaumur	Centigrade	Fahrenheit
−32	−40	−40
−16	−20	− 4
0	0	32
4	5	41
8	10	50
16	20	68
32	40	104
48	60	140
52	65	149
56	70	158
80	100	212

It will be noticed that the Reaumur degree is equivalent to 1.25 centigrade degrees.

Regulations.—Regulations concerning malt beverages are published by the Federal Alcohol Administration of the U.S. Tresury Department. The labeling and advertising of malt beverages, for example, are considered in Regulations 7 of the department.

Additional Information.—Further information concerning brewing may be obtained by referring to the publications listed at the end of the chapter. The texts of Hopkins and Krause; Hind; and Vogel, Schwaiger, Leonhardt, and Merten will be found to be of special value. "Bottle Beer Quality," a record of 10 years of research at the Wallerstein Laboratories, is also of particular interest.

Some Other Alcoholic Beverages.—Alcoholic beverages are consumed in every country of the world. In some countries the use of a particular beverage has been passed down from antiquity, for example, kvass in Russia, pulque in Mexico, tarete in Scandinavia, and sorgho in Manchuria. A brief description of a few alcoholic beverages follows:

Kvass may be prepared by mixing equal parts of barley malt, rye malt, and rye flour, adding boiling water, stirring, permitting the mash to stand for several hours, adding more boiling water, and then inoculating

with yeast and permitting fermentation to take place. Peppermint is added to the fermented product for flavoring. This beverage is very common in Russia. Neighboring countries prepare similar drinks to which other starchy or saccharine materials may be used.

Pulque is prepared by allowing the sweet juice of agave (the maguey plant is a common species) to undergo a spontaneous fermentation, which is usually complete in about 1 day. Yeasts produce alcohol from the sugars. Bacteria are present, which cause a rapid spoilage of the beverage unless it is consumed shortly after its manufacture. *Pulque* resembles cider and has a flavor somewhat similar to that of sour milk. It is a very common beverage in Mexico.

Tarbo is an alcoholic beverage prepared from milk. Yeasts cause the characteristic changes in flavor. The product has a pleasing acid taste.

Sorgho is an alcoholic drink made from *Sorghum saccharatum*.

Sake is the widely used alcoholic beverage of the Japanese. It is a yellow rice wine containing 14 to 24 per cent of alcohol. There are various methods by which it is manufactured. Usually a koji, known as *sake-koji* or *tane-koji*, is prepared. Steamed rice is inoculated with the spores of *Aspergillus oryzae* and incubated at about 30°C. until the rice is well covered with mycelium. The koji, or starter, is mixed with steamed rice and some water and incubated at a low temperature. Starch is converted to fermentable sugars. The thick liquid resulting from this enzyme hydrolysis and in which spontaneous fermentation usually takes place is known as *moto*. Koji, moto, and more water are usually mixed. An alcoholic fermentation ensues in which several yeasts may be active. *Saccharomyces sake*, *S. logos*, and *S. pastorianus* are some of the yeasts characteristic of sake.

Pombe is an alcoholic beverage made by permitting millet seed to sprout and undergo a conversion of the starch to sugars and by allowing a spontaneous fermentation of the saccharified starch in water.

Bili is a wine made from the tubercles of *Ophioglossum gramineum*. It is a West African drink.

Ginger beer is characterized by its distinctly acid nature, the ginger flavor, and the presence of a small amount of alcohol. Coffee decoctions 10 to 20 per cent concentration and pieces of ginger root "ginger-beer plant" is added to a solution of the sugar. In the ginger-beer plant are a yeast, *Saccharomyces pyriformis*, and a bacterium. *Bacterium vermiforme*. The yeast cells are entangled in the gelatinous sheaths of the bacteria. A symbiotic relationship apparently exists, for both the yeast and bacterium function best when in each other's presence.

Some American Brewing Periodicals

American Brewer (monthly), A. Schwarz Sons, New York, 1867–
American Brewers' Review (monthly), American Brewers' Review Co., Chicago, 1887–1937.
Brewers Bulletin (semiweekly), The Brewers Bulletin, Inc., Chicago, 1907–
Brewers Digest (formerly *Siebel Technical Review*) (monthly), Siebel Publishing Co., Chicago, 1925–
Brewers Journal (formerly *Western Brewer*) (monthly), H. S. Rich & Co., Chicago, 1876–
Brewery Age (monthly) Brewery Age Publishing Co., Chicago, 1932–
Modern Brewer (incorporated with *Brewer's Art*) (monthly), Modern Brewery, Inc., New York, 1920–
The West Coast Brewer (monthly), The West Coast Brewer, San Francisco, 1936–
ULRICH, C. F.: Periodicals Directory, 1938, R. R. Bowker Co., New York, 1938. (Contains a list of brewing periodicals.)

References on Brewing

ARNOLD, J. P., and F. PENMAN: "History of the Brewing Industry and Brewing Science in America," G. L. Peterson, Chicago, 1933.
ATKIN, L., and P. P. GRAY: Influence of Wort Constituents on the Rate of Fermentation, *Wallerstein Labs. Communs.* **10** (No. 31): 217–229 (1947).
BORUFF, C. S.: Industrial Wastes, Recovery of Fermentation Residues as Feeds, *Ind. Eng. Chem.*, **39** (No. 5): 602 (1947).
CHRZASZCZ, I., and J. JANICKI: Recent Advances in the Fermentation Industries, *Chem. and Ind.*, **55**: 884 (1936).
CLARK, W. M.: A Laboratory Mashing Apparatus, *Ind. Eng. Chem. (Anal. Ed.)*, **11**: 41 (1939).
COOPER, I. M.: "References Ancient and Modern to the Literature on Beer and Ale," United Brewers Industrial Foundation, New York, 1937.
COSBIE, A. J. C.: Brewing, the Story of a National Industry, *Wallerstein Labs. Communs.*, **5** (No. 15): 85–114 (1942).
DE CLERCK, J.: Papers concerned with oxidation-reduction in relation to brewing, *Wochschr. Brau.*, **51**: 196, 204, 213, 378 (1934); *Jour. Inst. Brewing*, **40**: 407, 417 (1934); *Am. Brewer*, **68** (January, 1935); *Modern Brewer*, **18** (No. 6): 38 (1937); *Modern Brewer*, **19** (No. 1): 40 (1938).
EHRNST, L. E.: Uses Laboratory to Direct Process, Assures Quality, *Food Ind.*, **11** (No. 2): 76 (1939).
GRAY, P. P., and I. STONE: Copper and Iron in Worts, Yeast and Beer, *Wallerstein Labs. Communs.*, **5** (No. 16): 193–199 (1942).
———, ———, and L. ATKIN: Systematic Study of the Influence of Oxidation on Beer Flavor, *Wallerstein Labs. Communs.*, **10** (No. 31): 183–194 (1947).
GUILLIERMOND, A.: "The Yeasts" (translated and revised by F. W. Tanner), John Wiley & Sons, Inc., New York, 1920.
HANES, C. S.: Starch Structure and Enzyme Action, *New Phytologist*, **36**: 101, 189 (1937).
HELM, E., and B. TROLLE: Experiments on Bottom Fermentation, I. Methodology, *Wallerstein Labs. Communs.*, **10** (No. 30): 87–98 (1947).
HIND, H. L.: Pasteur to 1936: An Account of the Development of Science in Brewing, *Chem.* and *Ind.*, **56**: 125 (1937).

——: "Brewing Science and Practice," Vol. I, "Brewing Materials," John Wiley & Sons, Inc., New York, 1938. (A second volume of this series is in preparation.)
Hopkins, R. H.: Prevention Better Than Cure for Brewing Troubles, *Food Ind.*, 10: 74 (1938).
——: Enzymes in Malting and Brewing, *Ann. Report*, 74 (No. 1): 66 (1937).
—— and C. B. Kraeer: "Biochemistry Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1937.
Jorgensen, A.: "Practical Management of Yeast Breeds," translated by Arthur Hansen, Charles Griffin & Company, Ltd., London, 1936.
Kolth, J. R., O. Nordeen, and L. Lampen: Comparison for Electron Determination of Proteolytic Activity of Malts, *Ind. Eng. Chem. (Anal. Ed.)*, 11: 35 (1939).
Lauer, S.: The Colorimetric Determination of Diastase and Dextrin and Its Application to Brewing Practice, *Am. Brewer* (December, 1935).
—— and R. Schwarz: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936.
Leach, A. E., and A. L. Winton: "Food Inspection and Analysis," 4th ed., John Wiley & Sons, Inc., New York, 1920.
Lewis, H.: The Significance of Oxygen in Brewing, *Brewers' Ann.*, 54 (No. 10): 366 (1937).
—— and C. Lyons: Acidity and Protein Turbidities in Beer Grains and Wort, *Lab. Carlsberg, Sér. chim.*, 22: 329 (1938).
Mees, R. H.: "Onderzoekingen over de Bierwaarden," Drukkerij Universitaire Pers, Groningen, 1934.
Mendlik, F.: Oxydations-Reduktionspotentiale im Bier, *Wochschr. Brau.*, 51: 307 (1934); *Am. Brewer*, 68: (December) (1935).
Nowak, C. A.: "Modern Brewing, A Practical Handbook of Contemporary Brewing Practice," 2d ed., Frank A. Roth Co., St. Louis, 1934.
——: "Twelve Treatises on Practical Brewing," Master Brewers' Assn., 1937.
Nuget, A. L.: "Brewing Formulas," Jersey Printing Co., Inc., Paterson, N. J., 1937.
Persons, W. M.: "Beer and Brewing in America, An Economic Study," United Brewers Industrial Foundation, New York, 1937.
Pozen, M. A.: Enzymes in Brewing, *Ind. Eng. Chem.*, 26: 1127 (1934).
Pyler, E. J.: Yeast Propagation in the Modern Brewery, *Brewers' Digest*, 14 (No. 2): 25 (1939).
Reich, G. T.: How to Convert Brewery to Industrial Alcohol Production, *Food Ind.*, 14 (No. 5): 46 (1942).
Schade, A. L.: Origin and Activity of Enzymes during Malting, *Communication to the Science and Practice of Brewing*, p. 13, December, 1937.
Schwarz, R.: Brewing Processes, *Ind. Eng. Chem.*, 27: 1031 (1935).
Shimwell, J. L.: Some Tests and Pollution in Brewing Investigation, *Journ. Inst. Brewing*, 44 (No. 12) (1938).
——: The Classification and Identification of Bacteria, with Special Reference to Beer Types, *Journ. Inst. Brewing*, 46 (No. 4): 291 (1940).
——: The Lactic Acid Bacteria of Beer, *Wallerstein Labs. Communs.*, 4 (No. 11): 41–48 (1941).
——: The Gram Stain and Its Significance in Brewing Practice, *Wallerstein Labs. Communs.*, 8 (No. 23): 23–33 (1945).
——: Yeast Contamination, *Brewers' Digest*, 22 (No. 4): 45T (1947).
Siebel, F. P., Jr.: Wartime Adjustments in Brewing Materials, *Master Brewers' Assn. Tech. Quart.*, 29 (No. 4): 29 (1945).

Sharp, Jr., P. F., and I. Sinclair: Application of Oxidation-Reduction Potential to Brewing Control, *Ind. Eng. Chem.*, **27**: 1042 (1935).
Shorter, L.: The History of American Brewing Literature, *Modern Brewer*, **19** No. 4: 40 (1938).
Stone, G. B.: Recent Advances in Brewing Technology, *Food Research*, **3**: 269 (1938).
Tressler, B.: Experiments on Bottom Fermentation. II. The Influence of Pitching Rate on Fermentation and on the Finished Beer, *Wallerstein Labs. Communs.*, **10** No. 30: 99–107 (1947).
United Brewers Industrial Foundation: "American Beer and Ale: A Handbook of Facts and Figures," New York.
U. S. Dept. of the Treasury, Federal Alcohol Administration: Federal Administration Act as in Effect on May 15, 1937.
U. S. Treas. Dept., *Annual Report of Commissioner of Internal Revenue for Fiscal Year* 1936, and, 1937.
Voigt, J. H., Jr., F. H. Schwalger, H. G. Leonhardt, and J. A. Merten: "The Practical Brewer," Master Brewers Association of America, St. Louis, 1946.
Walker, T. K.: A Review of Ten Years' Research on the Antiseptic Constituents of Hops, *Journ. Inst. Brewing*, **38**: 198 (1932).
Wallerstein, J. S.: The Role of Hops in Brewing, *Wallerstein Labs. Communs.*, **3** No. 8: 45–54 (1940).
Wallerstein Laboratories: *Communications on the Science and Practice of Brewing*, New York, December, 1937.
———— "Better Beer Quality," Wallerstein Laboratories, New York, 1947.
Wigand, J. K.: "A List of English, French, and German Periodicals on Brewing, Distilling, Wine and Other Alcoholic Beverages," The John Crerar Library, Chicago, 1933.
Wood, H. D.: "Hops, Their Production and Uses," The National Hops Company, Chicago, 1925.

Some Patents

Crown, R. S.: Coating Composition for Sheet Metal Containers, U.S. Patent 2,167,638 Aug. 1, 1939.
Greenwood, M., and H. Haynes: Beer Manufacturer, U.S. Patent, 1,898,047, Feb. 21, 1933.
Gubay, P. P., and I. Stone: Beer and Method of Preparing Same, U.S. Patent 2,159,-985 May 30, 1939.
Hermans, I.: Malt-Enzyme Extraction in Beer Making, U.S. Patent 2,163,200, June 20, 1939.
King, H. J. H.: Malt House, U.S. Patent 2,150,769, Mar. 14, 1939.
Lindner, R. L.: Process of Manufacturing Malt, U.S. Patent 2,137,141, Nov. 15, 1938.
Rose, C.: Process for Making Hopped Beer Work, U.S. Patent 2,163,468, June 20, 1939.
Schoch, P. G.: Lacquer Machine, U.S. Patent 2,150,009, Mar. 7, 1939.
Wilson, J. L., and W. L. Morgan: Process for Preparing a Brewing Adjunct, U.S. Patent 2,168,670, Jan. 17, 1939.

CHAPTER VII

WINE

Wine is the product made by the "normal alcoholic fermentation of the juice of sound, ripe grapes, and the usual cellar treatment."[1]

Classifications and Definitions.—Wines may be classified in many ways.

Dry wine is "wine in which the fermentation of the sugars is practically complete."[1] Most dry wines contain a small amount of sugar even though the quantity may be so slight as to escape detection by the sense of taste.

Sweet wine is "wine in which the alcoholic fermentation has been arrested."[1] Such wines contain sufficient sugar for taste perception. Wines may be fortified by the addition of brandy or wine spirits.

Fortified dry wine is "dry wine to which brandy has been added but which conforms in all other particulars to the standard of dry wine."[1]

Fortified sweet wine is "sweet wine to which wine spirits have been added."[1]

Sparkling wine is "wine in which the after part of the fermentation is completed in the bottle, the sediment being disgorged and its place supplied by wine or sugar liquor and/or dextrose liquor, and which contains, in 100 cc. (20°C.), not less than 0.12 g. of grape ash."[1] Such wine contains considerable carbon dioxide.

Wines may be red or white wines. A red wine is one containing the red coloring matter extracted from the skins of the grapes; a white wine is one "made from white grapes or the expressed fresh juice of other grapes."[2] In making a red wine, the skins and seeds are usually left with the must during fermentation.

"Modified wine, ameliorated wine, corrected wine is the product made by the alcoholic fermentation, with the usual cellar treatment of a mixture of the juice of sound, ripe grapes with sugar and/or dextrose, or a sirup containing not less than 65 per cent of the sugars, and in quantity not more than enough to raise the alcoholic strength after fermentation to 11 per cent by volume."[2]

[1] U.S. Dept. of Agriculture, F. D. A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

[2] *Ibid.*

Raisin wine is the "product made by the alcoholic fermentation of an infusion of dried or evaporated grapes, or of a mixture of such infusion or of raisins with grape juice."[1]

Regions of Production.—A large part of the world's wine is produced in the countries located near the Mediterranean Sea. France leads the world in the manufacture of wine, followed by Italy and Spain. Portugal, Greece, the Balkan States, and Germany; Algeria and other regions of North Africa; Chile and Argentina; Australia, Canada, and the United States produce considerable quantities of wine.

There are three principal regions of wine production in the United States, represented by (1) California; (2) Louisiana, Arkansas, and Missouri; and (3) New York, Ohio, New Jersey, and Michigan.[2]

Imports.—The imports of sparkling and still wines with their respective values for the years indicated are shown in Table 42.

TABLE 42.—AMOUNT AND VALUE OF IMPORTS OF SPARKLING AND STILL WINES[1,2]

	Quantity, thousands of gallons			Value, thousands of dollars		
	1934	1936	1947	1934	1936	1947
Sparkling wines	395	502	242	2,971	3,444	2,167
Still wines	3,463	3,134	1,919	10,212	8,021	6,643

[1] *U.S. Dept. of Commerce*, Statistical Abstract of the United States, 1937.

[2] The 1947 data were obtained from *U.S. Bur. Census, Foreign Trade Report* 110, U.S. Imports for Consumption of Merchandise, 1947.

During the calendar year 1947, the United States imported 181,974 gal. of Champagne, worth $1,610,373, chiefly from France and Italy; and 858,281 gal. of Vermouth, worth $2,497,035, principally from Italy, France, and Argentina. In addition, large quantities of grape wine were imported, particularly from Europe and South America.

Production Statistics.—Table 43 gives the production of still wine and the number of bonded wineries in the seven leading states and the United States during the fiscal years 1937 and 1946.

A perusal of this partial table indicates that California produces most of the still wine manufactured in the United States. New York follows California as the second most important state.

Sparkling wine production by eight leading states during the fiscal years 1937 and 1946 is as indicated in Table 44.

[1] *Ibid.*

[2] GORESLINE, H. E., Notes on Wine Manufacture in the United States, *U.S. Dept. of Agr.*, mimeographed sheets, 1936.

TABLE 43.—PRODUCTION OF STILL WINE AND NUMBER OF BONDED WINERIES IN LEADING STATES AND IN THE UNITED STATES, FISCAL YEARS 1937 AND 1946[1]

State	Production, wine gallons[2]		Bonded wineries[3]	
	1937	1946	1937	1946
California	115,338,166	365,008,818	630	413
New York	3,147,822	4,650,147	123	113
Washington	956,860	3,094,395	35	23
Oregon	.....	2,873,479	.....	22
Michigan	479,609	925,263	11	11
Ohio	755,175	752,539	130	102
Georgia	334,815	600,866	12	4
Total, United States	122,045,241	379,935,981	1,206	880

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1937 and 1946.

[2] Includes distilling materials (substandard wines produced with excessive water or residue materials).

[3] Number operated during any part of the year.

TABLE 44.—PRODUCTION OF SPARKLING WINE, FISCAL YEARS 1937 AND 1946[1]

State	Production, half-pint units	
	1937	1946[2]
New York	4,652,321	19,276,246
California	1,531,358	13,664,356
New Jersey	1,502,366	3,918,110
Missouri	984,022	1,447,738
Ohio	736,705	1,677,353
Michigan	171,745	432,996
Washington	41,312	
Pennsylvania	2,696	
Wisconsin	.....	152,589
Total, United States	9,622,525	40,569,388

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1937 and 1946.

[2] Includes production of 661,386 units of artificially carbonated wines.

Chemical Composition of Wines.—In Table 45 are shown the chemical analyses of some wines of American origin made by wine makers receiving awards at the Paris Exposition during the year 1900. The data presented in the table were compiled and computed from the analyses given by Wiley. For further details, the reader is referred to the publications of Amerine (1947), Amerine and Joslyn (1940), Joslyn and Amerine (1941), and Amerine and Winkler (1944).

Volatile Acids.—Acetic and propionic acids are the volatile acids found in sound wines. Acetic acid is the principal volatile acid of young wines,

TABLE 45.—CHEMICAL ANALYSES OF AMERICAN WINES RECEIVING AWARDS AT PARIS IN 1900[1]
(Grams per 100 cubic centimeters)

	Sparkling wines			Dry white wines			Dry red wines			Sweet white wines			Sweet red wines		
	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average
Specific gravity at 15.5°C	1.0169	0.9910	1.0045	0.9939	0.9901	0.9917	0.9969	0.9926	0.9943	1.0494	0.9908	1.0298	1.0522	1.0107	1.0276
Alcohol by volume, per cent	15.20	11.65	13.22	14.25	10.60	12.45	15.40	10.10	12.61	21.55	11.60	18.38	22.05	13.70	19.30
Alcohol	12.06	9.25	10.48	11.31	8.41	9.88	12.22	8.01	10.00	17.10	9.21	14.58	17.50	10.87	15.31
Glycerol	0.7330	0.2301	0.4177	1.0119	0.5812	0.7019	0.9504	0.5341	0.6355	0.7350	0.0483	0.3025	0.7460	0.2936	0.5089
Glycerol-alcohol ratio	.....	.....	.....	9.2:100	5.7:100	7.1:100	81:100	5.8:100	6.4:100						
Extract	8.56	1.78	5.40	2.51	1.55	1.98	3.22	1.77	2.57	19.45	2.83	13.80	19.71	7.57	13.52
Ash	0.290	0.114	0.153	0.270	0.107	0.196	0.393	0.138	0.247	0.263	0.097	0.203	0.374	0.234	0.311
Total acids	0.783	0.601	0.658	0.715	0.433	0.586	0.901	0.454	0.649	0.805	0.160	0.412	0.826	0.397	0.502
Fixed acids	0.715	0.418	0.566	0.570	0.326	0.459	0.690	0.346	0.507	0.528	0.116	0.300	0.508	0.257	0.472
Volatile acids	0.148	0.049	0.082	0.174	0.050	0.101	0.266	0.071	0.128	0.222	0.029	0.092	0.255	0.080	0.122
Total tartaric acid	0.357	0.163	0.271	0.352	0.119	0.189	0.252	0.083	0.163	0.296	0.038	0.142	0.145	0.025	0.078
Free tartaric acid	0.141	0.000	0.065	0.1785	0.0000	0.0677	0.0468	0.000	0.000	0.1436	0.000	0.0166	0.000	0.000	0.000
Polarization, °V	−13.3	−1.8	−5.2	−1.1	−0.1	−0.5	−0.8	0.0	−0.4	−25.7	0.0	−11.8	−19.4	−7.7	−16.4
Reducing sugars	5.584	0.023	3.409	0.328	0.051	0.134	0.280	0.045	0.146	16.910	1.767	11.30	16.960	3.240	10.26
Protein	0.698	0.070	0.214	0.337	0.060	0.162	0.305	0.077	0.150	0.245	0.026	0.162	0.368	0.105	0.232
Potassium sulphate	0.128	0.014	0.033	0.13	0.043	0.087	0.133	0.016	0.070	0.088	0.007	0.044	0.062	0.024	0.048
Phosphoric acid	0.026	0.012	0.017	0.066	0.010	0.022	0.044	0.015	0.029	0.059	0.008	0.034	0.068	0.018	0.045
Free sulphurous acid	0.0031	.....	.....	0.0078	0.0002	0.0017	.....	.....	.....	0.0059					
Total sulphurous acid	0.0456	0.0010	0.0074	0.0163	0.0013	0.0063	.....	.....	.....	0.0188	0.0011	0.0045	0.0047		
Tannin	0.066	0.009	0.035	0.0830	0.0178	0.0391	0.3435	0.1388	0.2364	0.0662	0.0213	0.0363	0.2207	0.0516	0.0962

[1] WILEY, H. W., American Wines at the Paris Exposition of 1900: Their Composition and Character, *U.S. Dept. Agr., Bur. Chem., Bull.* 72, 1903.

but old wines contain traces of propionic acid in addition.[1] Formic acid is usually found in diseased wines, together with acetic acid.

Fixed Acids. —Tartaric, malic, and tannic acids are constituents of the must and are, therefore, present in the wine in varying proportions. Much of the tartaric acid is removed during the manufacture of wine, especially during the fining process.[2]

Citric acid[3] is found in some grapes and in some wines. Succinic acid is produced in very small quantities during yeast fermentation. Phosphoric acid is a natural constituent of wine.

Wine Manufacture.—Although the fundamentals of wine making are similar in all three of the principal regions in the United States, methods vary to some extent in the different regions owing to the varieties of grapes used, the climate, and other factors. For example, a lower temperature may be used in the pasteurization of wines produced in the eastern portion of the United States on account of the greater acidity of the wine.

In view of the magnitude of the industry in California, special attention will be devoted to discussing wine making as it is practiced there, illustrating with a description of the production of red wine.

The reader desirous of securing additional knowledge of wine making will find numerous references at the end of this chapter, which should prove to be both interesting and instructive.

The Manufacture of Red Wine. *Outline of the Process.*—Selected grapes of the proper maturity are crushed and stemmed; treated with sulphur dioxide, or a sulphite, or pasteurized; and inoculated with a starter containing a pure culture of yeast. After a short fermentation period the wine is drawn off, placed in storage tanks for further fermentation, racked, stored for aging, clarified, and packaged.

Details of the Process. 1. THE GRAPE.—The production of a fine wine may be regarded as commencing with the selection of the best variety of grape for use in its manufacture. Bioletti[4] recommends the following varieties of grapes for the production of red wine: Beclan, Blue Portuguese, Cabernet Sauvignon, Carignane, Gros Verdot, Merlot, Mondeuse, Petite Sirah, Serine, Tannat, and Zinfandel for the coast counties of California; and Barbera, Grenache, Gros Mansenc, Lagrain, Pagadebito, Refosco, St. Macaire and Valdepēnas for the interior valleys of California.

[1] MORRIS, M. M., Volatile Acids of Wine, *Ind. Eng. Chem.*, **27**, 1250 (1935).

[2] WOODMAN, A. G., "Food Analysis," 3d ed., McGraw-Hill Book Company, Inc., New York, 1931.

[3] WINTON, A. L., and K. B. WINTON, "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.

[4] BIOLETTI, F. T., *Calif. Agr. Ext. Circ.* 30, Revised, 1934.

Amerine and Winkler[1] recommend the varieties shown in Table 45a for regions in California. The relative value of some white and red varieties of grapes for table and dessert wines is demonstrated in Table 45b.

The quality of the grapes of a given variety will depend upon the conditions under which they are grown—soil, climate, and other conditions.

Grapes should be gathered at the proper stage of maturity. In order to determine the degree of maturity, representative bunches of grapes are picked, and the Balling degree of their juice is determined. A reading of 21 to 23° Balling is usually given by the juice of the grapes when they are at the optimum stage of maturity.[2]

2. HANDLING THE GRAPES.—In gathering the grapes and transporting them to the winery, the prime purpose should be to have them arrive in the very best condition possible. The grapes should be picked with care, placed in clean containers, and protected from deterioration. Careful supervision of the handling of grapes is essential.

3. CRUSHING THE GRAPES.—Grapes are crushed and stemmed by machine. The chemical composition of the metal used in the construction of this machinery and other equipment about the winery is important. Iron and steel are used in some wineries but are undesirable for they may cause clouding of the wine, forming so-called "ferric casse." The tin and copper dissolved from bronze by grape juice, if sufficient in quantity, may cause flavor and color losses during the aging process. Stainless steel, nickel or inconel should be used in preference to iron, ordinary steel, and many bronzes.

If the grapes are not picked when cool, it is desirable to permit them to cool overnight before they are crushed.

4. TREATMENT BEFORE FERMENTATION.—Grapes contain on their surfaces a varied flora of microorganisms—molds, yeasts, and bacteria. It is quite possible that the juice of crushed grapes will produce a good wine without any special precautions, but a wine manufacturer cannot afford to gamble in respect to the quality of his wine. He can do much to ensure the quality of his final product by destroying or inhibiting the development of the microorganisms found on the grapes and by the use of starters containing pure cultures of the specific yeast desired.

Sulphur dioxide or sulphites destroy or inhibit the growth of many undesirable types of microorganisms—acetic acid bacteria, wild yeasts, and molds—with a minimum amount of injury to the true wine yeast. Usually 2 to 6 oz.,[3] or twice the quantity of potassium metabisulphite, are added per ton of crushed grapes, the quantity used depending on the

[1] AMERINE, M. A., and A. J. WINKLER, *Hilgardia*, **15** (No. 6): 493 (1944).
[2] JOSLYN, M. A., and W. V. CRUESS, *Calif. Agr. Ext. Circ.* 88, November, 1934.
[3] *Ibid.*

TABLE 45*a*.—RECOMMENDED VARIETIES OF GRAPES FOR REGIONS IN CALIFORNIA[1,2]

Region 1 (less than 2,500 degree-days)[3]	Region 2 (2,501 to 3,000 degree-days)[3]	Region 3 (3,001 to 3,500 degree-days)[3]	Region 4 (3,501 to 4,000 degree-days)[3]	Region 5 (4,001 or more degree-days)[3]
White Wines				
High-quality dry table wines: White Riesling Chardonnay Sauvignon blanc	*High-quality dry table wines:* Pinot blanc White Riesling Sauvignon blanc *Standard, special, or blending dry table wines:* Semillon Sylvaner Folle blanche	*Natural sweet wines:* Semillon Sauvignon blanc Muscat Canelli *Standard, special, or blending wines:* French Colombard Pinot blanc	*Standard-quality wines:* French Colombard *Muscatels:* Malvasia bianca Muscat Canelli Orange Muscat *Dessert wines:* Mission Palomino Verdelho	*Muscatels:* Malvasia bianca Orange Muscat *Dessert wines:* Palomino Mission Grenache
Red Wines				
High-quality table wines: Cabernet Sauvignon Pinot noir *Standard or blending wines:* Gamay Grenache	*High-quality table wines:* Cabernet Sauvignon *Standard, special or blending wines:* Grenache Petite Sirah Refosco	*Standard, special, or blending table wines:* Barbera Carignane Refosco *Dessert wine:* Trousseau	*Standard table wine:* Barbera *Bulk-quality table wines:* Carignane Refosco *Dessert wines:* Tinta Madeira Trousseau *Muscatel:* Aleatico	*Dessert wines:* Trousseau Tinta Madeira *Special or blending wines:* Salvador

See bottom of next page for footnotes.

TABLE 45A.—RELATIVE VALUE OF WHITE AND RED VARIETIES OF GRAPES FOR TABLE AND DESSERT WINES[1]

Table wines			Dessert wines		
Varieties	Relative value	Productivity	Varieties	Relative value	Productivity
White:			White:		
Chardonnay	92	Low	Palomino	82	High
White Riesling	88	Low	Muscat Canelli	82	Low medium
Sémillon	85	Medium	Orange Muscat	77	High
Sylvaner	85	Low medium	Gray Riesling	76	High
Pinot blanc	83	Low	Mulvasia bianca	73	Medium
Red:			Red:		
Cabernet Sauvignon	98	Low	Tinta Madeira	81	High medium
Pinot noir	92	Low	Mission	79	High
Grenache	82	High	Trousseau	77	High
Grignolino	79	Medium	Grenache	77	High
Barbera	78	High medium	Zinfandel	73	High medium
			Alicante	73	Medium

[1] Information obtained from M. A. Amerine and A. J. Winkler, Composition and Quality of Musts and Wines of California Grapes, *Hilgardia*, **15** (No. 6): 493-673 (1944). For information on additional varieties of lower relative value refer to article by Amerine and Winkler.

condition of the grapes—their maturity, the degree of contamination with molds, the temperature of the crushed product, and other factors. The largest quantities are used when the grapes are overripe, moldy, or relatively warm.

[1] Information obtained from article by M. A. Amerine and A. J. Winkler, *Hilgardia*, **15** (No. 6): 493-673 (1944).

[2] By this term is meant the summation of heat above 50°F. for the period of April to October, inclusive. For example, if the mean temperature for July was 75°F., the summation would be (75 − 50) 31 = 775 degree-days.

[3] Region 1. Napa and Oakville in Napa County, Hollister and San Juan Bautista in San Benito County; Woodside in San Mateo County; Mission San Jose in Alameda County; Saratoga in Santa Clara County; Paso Robles and Templeton in San Luis Obispo County; Soledad in Monterey County; and Guerneville, Santa Rosa, and Sebastopol in Sonoma County.

Region 2. Sunnyside in Monterey County; Rutherford, St. Helena, and Spring Mountain in Napa County; Santa Ynez Valley in Santa Barbara County; Almaden Vineyard, Evergreen, Guadalupe district, and Los Gatos in Santa Clara County; and Cloverdale in Sonoma County.

Region 3. Livermore and Pleasanton in Alameda County; Calpella, Ukiah, and Hopland in Mendocino County; Cucamonga in Napa County; Algiers in San Diego County; Templeton in San Luis Obispo County; Santa Ynez Valley in Santa Cruz County; and Alexander Valley, Asti, and Cloverdale in Sonoma County.

Region 4. Cucamonga in San Bernardino County; Escondido in San Diego County; Acampo, Escalon, Lockeford, Lodi, and Manteca in San Joaquin County; Cordelia in Solano County; Ceres, Hughson, and Vernalis in Stanislaus County; Ojai in Ventura County; and Davis in Yolo County.

Region 5. Livermore and Saticoy in Ventura County; Madera in Madera County; Arena and Livingston in Merced County; and Traver in Tulare County.[3]

[3] Quoted from M. A. Amerine and A. J. Winkler, Composition and Quality of Musts and Wines of California Grapes, *Hilgardia*, **15** (No. 6): 493-673 (1944).

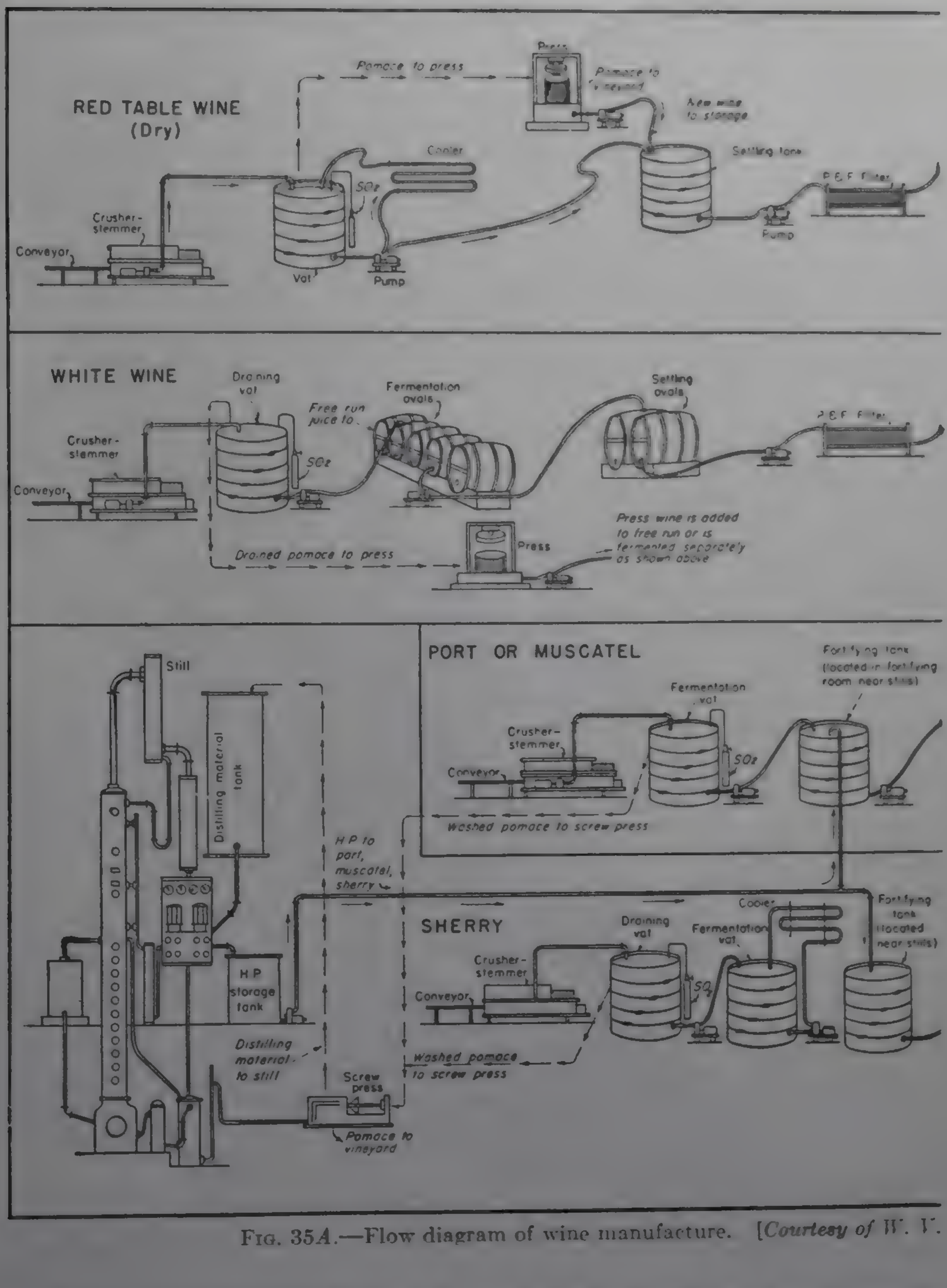
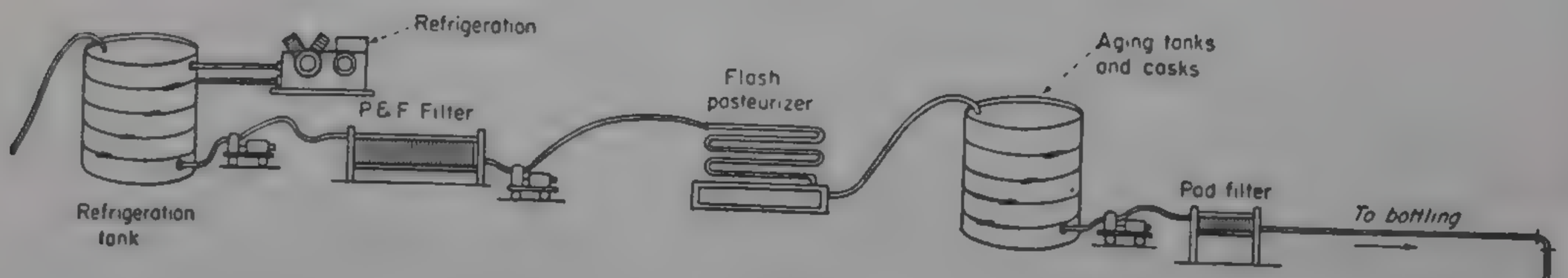
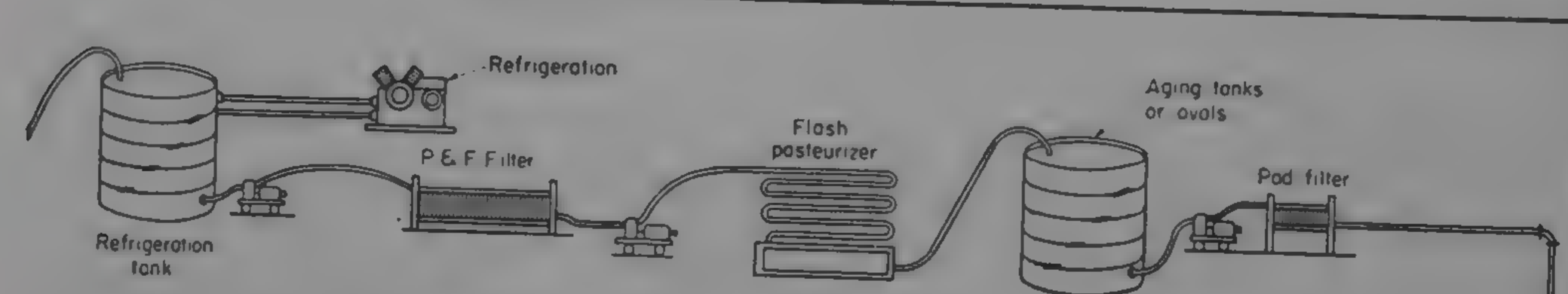


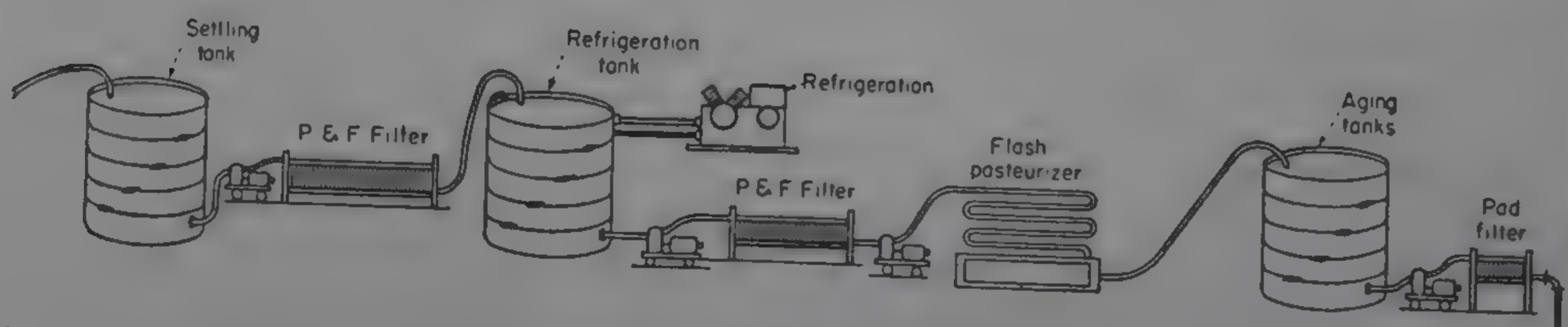
FIG. 35A.—Flow diagram of wine manufacture. [*Courtesy of* W. V.

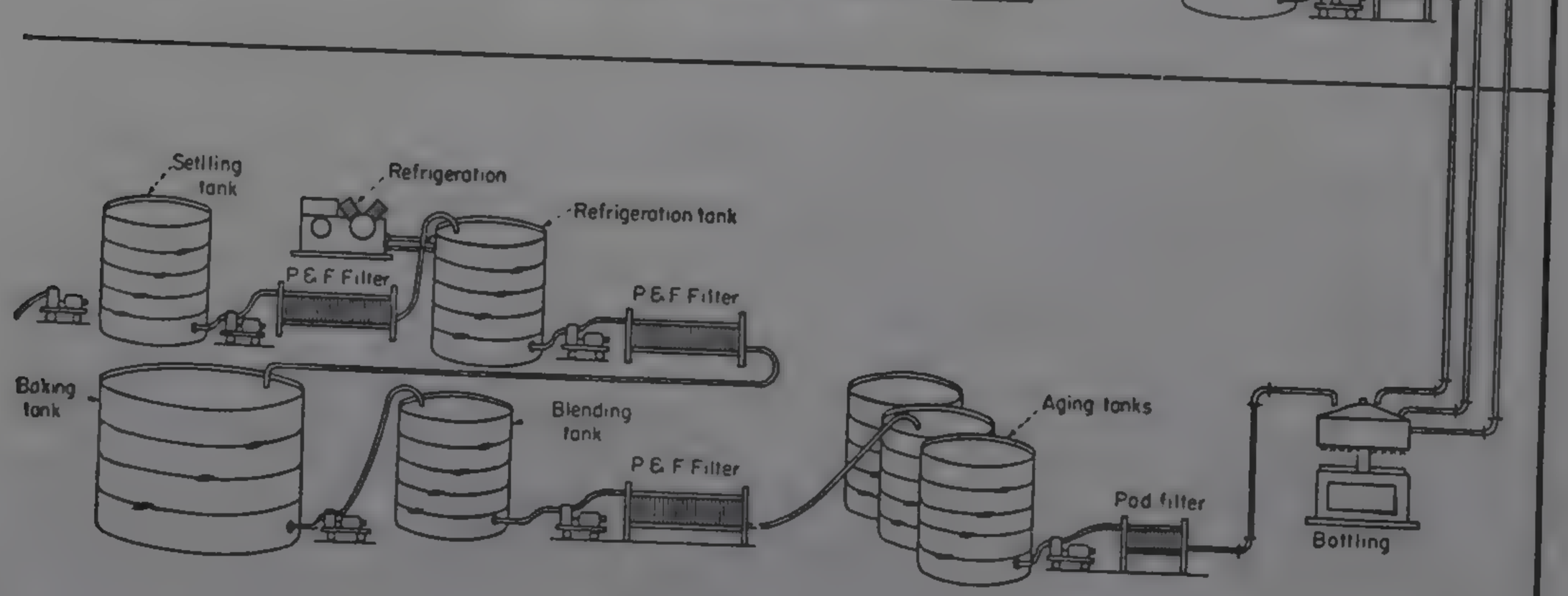
Pasteurization may be used in place of sulphites but is not usually considered to be so desirable.

5. THE FERMENTATION.—The selection of a yeast, the nutrient substances in the must (grape juice), the concentration of the sugar, the acidity, the oxygen supply, and the temperature are factors that must be supervised in respect to fermentation.









(*Cruess and C. R. Havighorst, Food Inds.*, **20** (No. 4): 523 (1948).]

Saccharomyces ellipsoideus is the yeast used for the fermentation of must. Selected strains, such as the Burgundy or Tokay strains, are frequently used. Many strains, bearing different names, are known.

A starter is prepared from a pure culture of the selected yeast. Pasteurized must is used as the culture medium in preparing the starter, the magnitude of which should represent 2 to 5 per cent of the volume of the crushed grapes being inoculated.

It is usually unnecessary to add any substances for the nutrition of the yeast since the crushed grapes are an adequate source of supply. On rare occasions ammonium sulphate or phosphates may be added.

Joslyn and Cruess[1] state that the optimum concentration of sugar is 22° Balling. The use of much higher concentrations of sugar favors the production of more than 13 per cent of alcohol by volume. Since alcohol tends to inhibit the fermentation when present in concentrations of 13 to 15 per cent by volume, a maximum of 13 per cent is usually desirable. The concentration that actually inhibits fermentation depends in part on the temperature of fermentation, the tolerance of the yeast for alcohol decreasing with increasing temperature. The approximate concentration of the alcohol that will be produced in the wine can be predetermined by multiplying the Balling reading of the must by 0.575.

It is permissible to reduce the concentration of sugar in must by the addition of water.[2] Another practice is to mix the juice with the high sugar concentration with a juice of low sugar concentration. Occasionally sugar may be added to must.

Grapes that have been permitted to become too mature are frequently of low acidity. Fruit acid—tartaric, citric, or malic acids—may be added to restore the normal acidity.

A large supply of oxygen is essential for the rapid multiplication of yeast cells and the starting of the fermentation, as stated under yeast manufacture (Chap. IX), while the later stage characterized by alcohol and carbon dioxide production rather than growth proceeds best under nearly anaerobic condition.

Approximately 6 hr. after treating the crushed grapes with sulphur dioxide or sulphite, the starter is added. Thereafter the contents of the tank are mixed, or stirred, at least twice a day, except during the main fermentation, to facilitate aeration, temperature equalization, and the extraction of color and tannin. Normally a "cap" forms on the surface of the fermentation vat, which contains grape skins, pieces of stem, seeds and other suspended matter. To mix the contents of the tank, one may punch down the cap or pump juice from the bottom of the vat over the surface of the must.

The amount of aeration produced by mixing the contents of the tank is determined by the effectiveness of the procedure and by the frequency at which the operation is repeated. Provided that the fermentation is slow at the beginning, or near the end of the incubation period, the supply of oxygen may be increased by more frequent mixing of the contents of

[1] *Ibid.*

[2] CRUESS, W. V., "The Principles and Practice of Wine Making," The Avi Publishing Co., Inc., New York, 1934.

the vat. However, the must should not be overaerated during fermentation, for overaeration is likely to produce a wine of inferior quality, especially insofar as color and flavor are concerned.

Fermentation should be carried out at carefully controlled temperatures. The finest wines are produced usually at temperatures below 85° F. (29.4° C.). The development of bouquet and aroma are favored by maintaining the fermenting must at low temperatures, around 70 to 75° F. (21.1 to 23.9° C.), for example. A temperature range of 70 to 90°F. (21.1 to 32.2° C.) is satisfactory. When the temperature rises to 85°F. (29.4° C.) or, at the most, to 90°F. (32.2° C.), the mash should be artificially cooled. Temperatures above 95°F. (35°C.) are considered unsafe, while the fermentation is inhibited usually at temperatures of 97 to 100°F. (36.1 to 37.8° C.). Fermentations cease at a temperature of 105°F. (40.5° C.)[3] generally. Undesirable bacteria develop at the higher temperatures. Accordingly the quality of the wine is impaired. Obviously, at too low temperatures, the fermentation is too slow to be practical.

During the fermentation, records of the temperature and the Balling degree should be made at least twice a day, one set of observations being recorded on the side of the fermentation vat in order that the progress of the wine may be followed.

After 3 to 5 days of active fermentation, sufficient tannin and a maximum of color have been extracted from the skin of the grape. Extraction is facilitated by the agitation of pomace (skin, seeds, and pieces of stems) during fermentation, by the ethyl alcohol produced from the grape sugar, the heat of fermentation, and the mechanical breaking up of the skin.

The wine maker decides when the color and tannin content are satisfactory and then draws off the wine to separate it from the pomace. He does not wait for all the sugar to be fermented. At the time of drawing off the wine, the Balling reading may be 0 to 4°. It is not considered advisable to mix the wine drawn off ("free-run wine") with that expressed from the pomace; for the latter is of lower quality.

6. FURTHER FERMENTATION.—The free-run wine is placed in closed storage tanks, equipped with bungs that allow the excess carbon dioxide to escape. An atmosphere of carbon dioxide over the wine tends to inhibit the development of acetic acid bacteria and other aerobic types of microorganisms. The fermentable sugar is usually consumed in 7 to 11 days at a temperature of 70 to 85° F. (21.1 to 29.4°C.).

If the after fermentation becomes sluggish before the sugar is utilized, the yeast may be activated by pumping over the wine.

7. RACKING.—By racking is meant the drawing off of the wine from the lees or sediment. Potassium bitartrate ($KHC_4H_4O_6$, *i.e.*, cream of

tartar, is found in the lees. This substance is less soluble in alcohol than in water and precipitates out more rapidly at low temperatures.

Wine is racked to facilitate its clearing and to prevent undesirable flavors from being extracted from the old yeast.

8. STORAGE AND AGING.—Two important changes take place during storage and aging: clearing of the wine and the development of flavor.

In a new wine there are present substances which, if not removed, will produce a sediment and probably cloudiness. These substances include tartrates, certain proteins, and other matter. Naturally these substances would be removed by racking and filtration during a somewhat long storage and aging process, but the modern trend is to hasten the removal of these substances by methods that involve flash pasteurization (to precipitate certain proteins), cooling to room temperature and then to 24 to 27°F. (−4.44 to −2.78°C.), and holding at the latter temperature for a few days. Filtration is carried out in the cold. Since the acid content of the wine is frequently reduced by the foregoing rapid process it is customary to adjust the acidity with citric or tartaric acids, the former acid being preferred. The wine is placed in tanks for aging.

Wine storage tanks are generally constructed of white oak or redwood, white oak being the better of the two. The tanks are completely filled with wine and sealed to prevent the access of large quantities of oxygen, which would favor the growth of acetic acid bacteria and *Mycoderma vini* (wine flowers). Some wine is always lost through evaporation. Consequently, the tanks should be inspected regularly and kept filled with wine. Periodically the wine is racked. During racking and filling, especially, carbon dioxide is lost while some oxygen is absorbed. A small amount of oxygen accumulates in the headspace over the wine.

Flavor, which is due to a combination of taste and odor, is developed in wine as a result of oxidative changes and ester formation.

Aging proceeds slowly until oxygen becomes available in small quantities. It is inhibited by the presence of large quantities of carbon dioxide, by sulphur dioxide, and by the exclusion of air. New wines stored in airtight bottles do not age properly.

Alcohols, aldehydes, tannins, and other substances present in the wine are oxidizable. Alcohols may be converted to aldehyde and subsequently to acids by oxidation. Aldehydes form acetals with alcohol.

Combinations of alcohols with acids give rise to esters, which are important in the production of aroma or bouquet. Although opinions differ concerning the importance of esters, it is recognized that the nature of volatile esters is of greater significance than the quantity.[1] The esters of acetic acid contribute much to the flavor and bouquet of wine.

[1] NELSON, E. K., *Food Research*, **2**: 221 (1937).

The time required for aging varies with the type of wine and the conditions. A dry wine may require at least 2 years. Some fine wines are aged for 5 or more years.

9. CLARIFICATION.—Wines may clear naturally over a period of time, but resort is frequently made to the use of finings followed by filtration, heating, refrigeration, or combinations of the foregoing. Fining agents, which include such substances as casein, gelatin and tannin, bentonite (of Wyoming origin, if possible), isinglass (fish protein from the sturgeon), white of egg, and Spanish clay, are mixed with the wine carefully according to direction, or preferably after laboratory tests have been carried out with small portions of the wine and the fining agents. The improper use of some of the fining agents may, in itself, be a cause of clouding of the wine.

Filtration is carried out with filter aids. For further details the reader is referred to "The Principles and Practice of Wine Making" by Cruess and to the more recent publications on this subject.[1]

10. PACKAGING.—The clarified wine is placed in oak barrels for bulk sale and in bottles or in cans for unit sale.

Bottles of small and medium size may be pasteurized for 30 min. at 140°F.[2]

Defects of Wine.—Defects of wine may be caused by microorganisms, in which case they are known as diseases, or by other agencies. The diseases of wine are of two general types: those caused by aerobic microorganisms, and those caused by facultative anaerobes or anaerobes.

Diseases Caused by Aerobic Microorganisms.—The aerobic diseases of wines are caused principally by mycodermas and acetic acid bacteria. These microorganisms grow well in wine in the presence of oxygen. They cause no trouble if the wine is carefully supervised during its manufacture and storage. They are most likely to become active during the fermentation of must, if the cap is not punched down frequently, and during the storage of wine, if the containers are not kept properly filled and sealed.

Mycoderma vini (wine flowers) forms a film over the surface of wine and attacks the extract, the alcohol, and occasionally the organic acids.

Acetic acid bacteria will produce vinegar from wine in the presence of oxygen, unless they are destroyed or prevented from growing. Their activities are discussed in the chapter on vinegar.

Diseases Caused by Facultative Anaerobes or Anaerobes. TOURNE DISEASE.—The term "tourne" is considered to signify either the organism causing the disease or the condition produced in wine by large numbers of

[1] SAYWELL, L. G., *Ind. Eng. Chem.*, **27**: 1245 (1935).
[2] CRUESS, W. V., *Fruit Products Jour.*, **15**: 40 (1935).

these bacteria.[1] Tourne is considered to be the most serious disease of wines and one of the most common.[2]

The organism is an anaerobic bacterium, which occurs as long, slender rods. It may be found in red or white wines, or in fortified wines with an alcohol content of 20 per cent or greater. It grows best, however, if the alcohol concentration is not too great. Sugar and other nutrient substances favor its growth. It is likely to develop in wines "stuck" due to high temperatures. It is inhibited slightly by tannin but very strongly by sulphur dioxide and metabisulphites.

Tourne is indicated[3] by increasing volatile acidity, by decreasing fixed acidity, by a "silky" type of cloudiness, and, when the condition has progressed well, by an odor and taste that is termed "mousey."

Tourne may be detected by a microscopic examination of the sediment, obtained by centrifuging a sample of the wine, or by analyses of the wine for volatile acids. If the maximum amount of volatile acid permitted in wine by law—0.14 for red wine or 0.12 for white wine—has been produced or exceeded, then there is good evidence that the wine may be infected with tourne. Taste may also be of some assistance in its detection.

The judicious application of sulphur dioxide, 75 p.p.m., to wines, or pasteurization; the use of a high degree of cleanliness about the plant; sterilization of equipment with steam when necessary; and rigid laboratory control should lower the incidence, or prevent, tourne disease of wine.

A pasteurization of bottled wine at a temperature of 145°F. for 30 min. is very effective in preventing tourne.

Once wine has been infected by tourne, it should be made brilliantly clear by filtration with selected infusorial earths, or by clarification with bentonite followed by passage through germproof filters.[4] Sulphur dioxide, or its equivalent of metabisulphite, should then be added to the wine in such quantity that its concentration will be maintained at 75 p.p.m., or greater. All equipment that has been infected should be treated with live steam or a suitable disinfectant to destroy the source of infection.

LACTOBACILLUS HILGARDII.—Spoilage of some dry wines in California has been caused by *Lactobacillus Hilgardii*,[5] a nonmotile, nonsporulating rod, which produces lactic and acetic acids. A silky cloudiness is produced, while the flavor is affected and becomes somewhat "mousey."

[1] CRUESS, W. V., The Tourne Disease of Wine, *Fruit Products Jour.*, **14**: 198 (1935).
[2] JOSLYN and CRUESS, *loc. cit.*
[3] JOSLYN and CRUESS, *loc. cit.*
[4] CRUESS, W. V., *Fruit Products Jour.*, **14**: 198 (1935).
[5] DOUGLAS, H. C., and W. V. CRUESS, *Food Research*, **10**: 113 (1936).

MANNITOL (MANNITE) FORMING BACTERIA.—Bacteria that produce volatile acid, lactic acid, and mannitol ($C_6H_{14}O_6$) from glucose grow well at temperatures above 100° F. Their growth is inhibited by keeping the temperature of fermentation well below this point, by the use of sulphur dioxide, and by an increase in the acid content of the wine due to the addition of citric or tartaric acid.[1]

SLIME FORMING BACTERIA.—Infected wine may become slimy and cloudy. Slime formation occurs chiefly in white wines, usually young wines in closed containers. The occurrence of slime-forming bacteria in wine is not common and may be prevented by adding tannin to wines low in this substance and by use of sulphur dioxide or metabisulphite.

OTHER BACTERIAL DISEASES.—Cocci, which may be inhibited by SO_2 or destroyed by pasteurization, may cause cloudiness in white wines.

Bitter wines may be caused by the growth of butyric acid bacteria, while a sour wine may result from the growth of lactic acid bacteria.

Defects Not Caused by Microorganisms.—Defects in wines may be caused by metals, enzymes, and the improper use of certain fining agents.

Iron is a cause of clouding in wines. Two different types of defects are produced by iron: one is known as black, blue, or ferric casse; the other as white casse.

Ferric casse is indicated by the appearance of a gray to gray-blue sediment in the wine and by clouding. The defect is found in bottled white wines, especially. Iron forms a precipitate with the tannin and coloring matter of red wine. Only a few parts of iron in a million parts of wine will produce ferric casse. Oxygen facilitates the formation of ferric ions from ferrous ions.

Ferric casse may be prevented by using equipment constructed of the proper types of metal and inhibited by 0.1 per cent citric acid.

The defect may be treated in one of several manners. In one method,[2] the iron is oxidized to the ferric stage by aeration. Tannin to the amount of 0.05 per cent is added. Clarification with casein and bentonite follow. In a second method, the iron is oxidized by aeration, tannin is added, and then gelatin. Settling is permitted, which is followed by racking, aeration, and acidification with citric acid. In a third method, tartaric acid is added to the wine, and the wine is then refrigerated. Cream of tartar and iron salts are precipitated.

White casse is also caused by an excess of iron. The precipitate is due in part to iron phosphate. It occurs in white wine. Treatment is as outlined above for ferric casse.

Oxidase casse is uncommon. It is caused by an enzyme, oxidase, produced by certain molds, which causes white wines to become brown

[1] Joslyn and Cruess, *loc. cit.*

[2] Sawyer, L. G., *Ind. Eng. Chem.*, **26**: 379 (1934).

and the color to be precipitated in red wines. Sulphur dioxide inhibits this oxidase, while pasteurization at 180°F. destroys it.

Other Defects.—Tin, tin salts, copper, aldehydes, or excessive quantities of gelatin may cause clouding of the wine. For a further discussion of this subject the reader is referred to the publications of Cruess and other workers.

Coatings of Wine Tanks.—Concrete tanks are used for the fermentation of must and the storage of wine, but they must be lined to prevent an undue amount of calcium from being dissolved by the wine. Steel tanks should be lined to prevent the solution of iron.

According to Cruess[1] and his associates one effective method for coating concrete is to treat the concrete first with a 0.5 per cent, then a 25 per cent, solution of tartaric acid. An insoluble calcium tartrate forms over the surface of the tank.

A mixture of 25 per cent Gilsonite and 75 per cent paraffin produces a protective coating for concrete and steel. Such linings are easily applied and not expensive.

Bass-Hueter black enamel gives good results[1] with both steel and concrete. A coating of paraffin over the enamel gives even better protection.

Standards of Identity for Wine.[2]—The Federal Alcohol Administration has set up standards of identity for wine, the text of which follows:

Article II. Sec. 20. *Application of standards.*—The standards of identity for the several classes and types of wine set forth herein shall be applicable to all regulations and permits issued under the act. Whenever any term for which a standard of identity has been established herein is used in any such regulation or permit, such term shall have the meaning assigned to it by such standard of identity.

Sec. 21. *The standards of identity.*—Standards of identity for the several classes and types of wine set forth herein shall be as follows:

Class 1. *Grape wine.*—(*a*) "Grape wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe grapes (including restored or unrestored pure condensed grape must), with or without the addition, after fermentation, of pure condensed grape must, and with or without added fortifying grape spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by either of the following methods:

(1) By adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent; but in no event shall any product so ameliorated have an

[1] Cruess, W. V., T. Scott, H. B. Smith, and L. M. Cash, *Food Research*, **2**: 385 (1937).

[2] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 4, Amendment 2, Washington, Aug. 22, 1938.

alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

(2) By adding, separately or in combination, not more than 11 per cent by weight of dry sugar, or not more than 10 per cent by weight of water.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural red wine, more than 0.14 gram, and for other grape wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Red wine" is grape wine which contains the red coloring matter of the skins, juice, or pulp of grapes.

(*c*) "White wine" is grape wine which does not contain the red coloring matter of the skins, juice, or pulp of grapes.

(*d*) "Light wine" (including "light grape wine," "light red wine," and "light white wine") is grape wine having an alcoholic content not in excess of 14 per cent by volume.

(*e*) "Natural grape wine" (including "natural red wine" and "natural white wine") is grape wine containing no fortifying grape spirits or added alcohol.

(*f*) "Angelica," "madeira," "muscatel," and "port" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to these products, and an alcoholic content of not less than 18 per cent by volume.

(*g*) "Sherry" is a type of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to this product, and an alcoholic content of not less than 17 per cent by volume.

(*h*) "Light port" and "light sherry" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to "port" and "sherry," respectively, and an alcoholic content of more than 14 per cent by volume.

Class 2. *Sparkling grape wine.*—(*a*) "Sparkling grape wine" (including "sparkling wine," "sparkling red wine," and "sparkling white wine") is grape wine made effervescent with carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

(*b*) "Champagne" is a type of sparkling light white wine which derives its effervescence solely from the secondary fermentation of the wine within glass containers of not greater than one gallon capacity, and which possesses the taste, aroma, and other characteristics attributed to champagne as made in the champagne district of France.

(*c*) A sparkling light white wine having the taste, aroma, and characteristics generally attributed to champagne but not otherwise conforming to the standard for "champagne" may, in addition to but not in lieu of the class designation "sparkling wine," be further designated as "champagne style" or "champagne type" or "American (or New York State, California, etc.) champagne—bulk process"; all the words in any such further designation shall be equally conspicuous and shall appear in direct conjunction with and in lettering approximately one-half the size of the words "sparkling wine."

Class 3. *Carbonated grape wine.*—(*a*) "Carbonated grape wine" including "carbonated wine," "carbonated red wine," and "carbonated white wine") is grape wine made effervescent with carbon dioxide other than that resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

Class 4. *Citrus wine.*—(*a*) "Citrus wine" or "citrus fruit wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe citrus fruit (including restored or unrestored pure concentrated citrus must), with or without the addition, after fermentation, of pure concentrated citrus must, and with or without added fortifying citrus spirits or alcohol, but without other additions or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an ameliorated residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural citrus wine, more than 0.14 grams, and for other citrus wine, more than 0.12 grams per 100 cubic centimeters (20° C.).

(*b*) "Light citrus wine" or "light citrus fruit wine" is citrus wine having an alcoholic content not in excess of 14 per cent by volume.

(*c*) "Natural citrus wine" or "natural citrus fruit wine" is citrus wine containing no fortifying citrus spirits or added alcohol.

(*d*) Citrus wine derived wholly (except for sugar, water, or added alcohol) from one kind of citrus fruit, shall be designated by the word "wine" qualified by the name of such citrus fruit, *e.g.*, "orange wine," "grapefruit wine." Citrus wine not derived wholly from one kind of citrus fruit shall be designated as "citrus wine" or "citrus fruit wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Citrus wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and citrus wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 5. *Fruit wine.*—(*a*) "Fruit wine" is wine (other than grape wine or citrus wine) produced by the normal alcoholic fermentation of the juice of sound, ripe fruit (including restored or unrestored pure concentrated fruit must), with or without the addition, after fermentation, of pure concentrated fruit must, and with or without added fortifying fruit spirits or alcohol, but without other additions or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived

by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural fruit wine, more than 0.14 gram, and for other fruit wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Berry wine" is fruit wine produced from berries.

(*c*) "Light fruit wine" is fruit wine having an alcoholic content not in excess of 14 per cent by volume.

(*d*) "Natural fruit wine" is fruit wine containing no fortifying fruit spirits or added alcohol.

(*e*) Fruit wine derived wholly (except for sugar, water, or added alcohol) from one kind of fruit shall be designated by the word "wine" qualified by the name of such fruit, *e.g.*, "peach wine," "blackberry wine." Fruit wine not derived wholly from one kind of fruit shall be designated as "fruit wine" or "berry wine," as the case may be, qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Fruit wines which are derived wholly (except for sugar, water, or added alcohol) from apples or pears may be designated "cider" and "perry," respectively, and shall be so designated if lacking in vinous taste, aroma, and characteristics. Fruit wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and fruit wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 6. *Wine from other agricultural products.*—(*a*) Wine of this class is wine (other than grape wine, citrus wine, or fruit wine) made by the normal alcoholic fermentation of sound fermentable agricultural products, either fresh or dried, or of the restored or unrestored pure condensed must thereof, with the addition before or during fermentation of a volume of water not greater than the minimum necessary to correct natural moisture deficiencies in such products, with or without the addition, after fermentation, of pure condensed must, and with or without added alcohol or such other fortifying spirits as will not alter the character of the product, but without other addition or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural wine of this class, more than 0.14 gram, and for other wine of this class, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Light" wine of this class is wine having an alcoholic content not in excess of 14 per cent by volume.

(*c*) "Natural" wine of this class is wine containing no fortifying spirits or added alcohol.

(*d*) "Raisin wine" is wine of this class made from dried grapes.

(*e*) "Sake" is wine of this class produced from rice in accordance with the commonly accepted method of manufacture of such product.

(*f*) Wine of this class derived wholly (except for sugar, water, or added alcohol) from one kind of agricultural product shall, except in the case of "sake," be designated by the word "wine" qualified by the name of such agricultural product, *e.g.*, "honey wine," "raisin wine," "dried blackberry wine." Wine of this class not derived wholly from one kind of agricultural product shall be designated as "wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Wine of this class rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and wine of this class rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 7. *Vermouth.*—(*a*) "Vermouth" is a compound having an alcoholic content of not less than 15 per cent by volume, made by the mixture of extracts from macerated aromatic flavoring materials with grape wine containing fortifying grape spirits or added alcohol, and manufactured in such a manner that the product possesses the taste, aroma, and characteristics generally attributed to vermouth.

Class 8. *Imitation, concentrate, and substandard wine.*—(*a*) "Imitation wine" shall bear as a part of its designation the word "imitation," and shall include:

(1) Any wine containing synthetic materials.

(2) Any wine made from a mixture of water with residue remaining after thorough pressing of grapes, fruit, or other agricultural products.

(3) Any class or type of wine the taste, aroma, color, or other characteristics of which have been acquired in whole or in part, by treatment with methods or materials of any kind, if the taste, aroma, color, or other characteristics of normal wines of such class or type are acquired without such treatment.

(*b*) "Concentrate wine" shall bear as a part of its designation the word "concentrate," and shall include any wine made from must concentrated at any time to more than 80° (Balling).

(*c*) "Substandard wine" shall bear as a part of its designation the word "substandard." and shall include:

(1) Any wine having a volatile acidity in excess of the maximum prescribed therefor in this article.

(2) Any wine for which no maximum volatile acidity is prescribed in this article, having a volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, in excess of 0.14 gram per 100 cubic centimeters (20°C.).

(3) Any wine for which a standard of identify is prescribed in this article, which, through disease, decomposition, or otherwise, fails to have the com-

sary to correct cloudiness, precipitation, or abnormal color, odor, or flavor developing in wine.

(5) Treatment with constituents naturally present in the kind of fruit or other agricultural product from which the wine is produced for the purpose of correcting deficiencies of these constituents, but only to the extent that such constituents would be present in normal wines of the same class or type not so treated.

Sec. 23. *Grape type designations.*—(*a*) A name indicative of a variety of grape may be employed as the type designation of a grape wine if the wine derives its predominant taste, aroma, and characteristics, and at least 51 per cent of its volume, from that variety of grape. If such type designation is not known to the consumer as the name of a grape variety, there shall appear in direct conjunction therewith an explanatory statement as to the significance thereof.

Sec. 24. *Generic, semi-generic, and non-generic designations of geographic significance.*—(*a*) A name of geographic significance which is also the designation of a class or type of wine, shall be deemed to have become generic only if so found by the Administrator.

Examples of generic names, originally having geographic significance, which are designations for a class or type of wine are: vermouth, sake.

(*b*) A name of geographic significance, which is also the designation of a class or type of wine, shall be deemed to have become semi-generic only if so found by the Administrator. Semi-generic designations may be used to designate wines of an origin other than that indicated by such name only if there appears in direct conjunction therewith an appropriate appellation of origin disclosing the true place of origin of the wine.

Examples of semi-generic names which are also designations for types of grape wine are: Angelica, Burgundy, Claret, Chablis, Champagne, Chianti, Malaga, Marsala, Madeira, Moselle, Port, Rhine Wine (syn. Hock), Sauterne, Haut Sauterne, Sherry, Tokay.

(*c*) A name of geographic significance, which has not been found by the Administrator to be generic or semi-generic, may be used only to designate wines of the origin indicated by such name, but such name shall not be deemed to be the distinctive designation of a wine unless the Administrator finds that it is known to the consumer and to the trade as the designation of a specific wine of a particular place or region, distinguishable from all other wines.

Examples of non-generic names which are not distinctive designations of specific wines are: American, California, Lake Erie Islands, Napa Valley, New York State, French, Spanish.

Examples of non-generic names which are also distinctive designations of specific grape wines are: Bordeaux Blanc, Bordeaux Rouge, Graves, Medoc, St. Julien, Chateau Yquem, Chateau Margaux, Chateau Lafite, Pommard, Chambertin, Montrachet, Rhone, Liebfraumilch, Rudesheimer, Forster Deidesheimer, Schloss Johannisberger, Lagrima, Lacryma Christi.

Sec. 25. *Appellations of origin.*—(*a*) A wine shall be entitled to an appellation of origin if (1) at least 75 per cent of its volume is derived from fruit or other agricultural products both grown and fermented in the place or region indicated

by such appellation, (2) it has been fully manufactured and finished within such place or region, and (3) it conforms to the requirements of the laws and regulations of such place or region governing the composition, method of manufacture, and designation of wines for home consumption.

(*b*) Wines subjected to cellar treatment outside the place or region of origin under the provisions of section 22(*c*) of this article, and blends of wines of the same origin blended together outside the place or region of origin (if all the wines in the blend have a common class, type, or other designation which is employed as the designation of the blend), shall be entitled to the same appellation of origin to which they would be entitled if such cellar treatment or blending took place within the place or region of origin.

References on Wine

ADAMS, F. E.: Chemical Sterilization in the Winery, *Fruit Products Jour.*, **13**: 177 (1934).

ALLEN, H. W.: "The Wines of France," T. Fisher Unwin, London, 1924.

———: "The Romance of Wine," E. P. Dutton & Company, New York, 1932.

AMERINE, M. A.: Determination of Esters in Wines—Liquid-Liquid Extraction, *Food Research*, **9** (No. 5): 392–395 (1944).

———: The Composition of California Wines at Exhibitions, *Wines & Vines*, **28** (No. 1): 21–23, 42–43, 45; (No. 2): 24–26; (No. 3): 23–25, 42–46 (1947).

——— and W. DEMATTEI: Color in California Wines, III. Methods of Removing Color from the Skins, *Food Research*, **5** (No. 5): 509–519 (1940).

——— and W. C. DIETRICH: Glycerol in Wines, *Jour. Assoc. Offic. Agr. Chemists*, **26** (No. 3): 408–413 (1943).

——— and M. A. JOSLYN: Commercial Production of Table Wines, *Bull.* 639, *Univ. Calif.*, July, 1940.

———, L. P. MARTINI, and W. DEMATTEI: Foaming Properties of Wine, *Ind. Eng. Chem.*, **34**: 152 (1942).

——— and A. D. WEBB: Alcohol-Glycerol Ratio of California Wines, *Food Research*, **8** (No. 4): 280–285 (1943).

——— and A. J. WINKLER: Maturity Studies with California Grapes I. The Balling-Acid Ratio of Wine Grapes, *Proc. Am. Soc. Hort. Sci.*, **38**: 379-387 (1940).

——— and ———: Maturity Studies with California Grapes II. The Titratable Acidity, pH, and Organic Acid Content, *Proc. Am. Soc. Hort. Sci.*, **40**: 313-324 (1942).

——— and ———: Color in California Wines IV. The Production of Pink Wines, *Food Research*, **6** (No. 1): 1–14 (1941).

——— and ———: Composition and Quality of Musts and Wines of California Grapes, *Hilgardia*, **15** (No. 6): 493–673 (1944).

ARNULF, F.: "The New Wine Book. The Biology of Wine Fermentation and Its Practical Importance," Western Printing Corp., Whittier, California, 1936.

ASH, C. S.: Metals in Wineries, *Ind. Eng. Chem.*, **27**: 1243 (1935).

Association of Official Agricultural Chemists: "Official and Tentative Methods of Analysis," 6th ed., Washington, D. C., 1945.

BABO, A. F. VON, und E. MACH: "Handbuch des Weinbaues und der Kellerwirtschaft," 4 vols. Paul Parey, Berlin, 1923–1927.

BEAVENS, E. A., H. E. GORESLINE and C. S. PEDERSON: Preservation of Grape Juice, *Food Research*, **3**: 467 (1938).

BIGELOW, W. D.: The Composition of American Wines, *U.S. Dept. Agr., Bur. of Chem., Bull.* 59, 1900.

BIOLETTI, F. T.: Conditioning Wine Containers, *Calif. Grape Grower*, **24**: 25 (1934).

———: "Wine Making on a Small Scale," *Calif. Grape Grower*, San Francisco, 1934.

BOSWELL, P.: "Wine Makers Manual," Orange Judd Publishing Co., Inc., New York, 1935.

BOUTARIC, A., L. FERRÉ, et M. ROY: Recherches spectrophotométriques sur la couleur des vins, *Ann. Fals.*, **30**: 196 (1937).

BROWN, E. M., and V. DEF. HENRIQUES: Vinification in California Wineries, *Ind. Eng. Chem.*, **27**: 1235 (1935).

BURDONI, M.: "Trattato di Enologia," F. Natelli Strini, Albano Laziale, Italy, 1930.

BUTLER, F. H.: "Wine and Wine Lands of the World," Coward-McCann, Inc., New York, 1926.

BYRNE, J. L., L. G. SAYWELL, and W. V. CRUESS: The Iron Content of Grapes and Wine, *Ind. Eng. Chem.* (*Anal. Ed.*), **9**: 83 (1937).

CASH, L.: The Use of Inert Gas or Mineral Oil in the Storage of Wines, *Fruit Products Jour.*, **16**: 4 (1936).

CHAMPLIN, F. M., H. E. GORESLINE, and D. K. TRESSLER: Manufacture of Champagne and Sparkling Burgundy, *Ind. Eng. Chem.*, **27,** 1240 (1935).

CHARLEY, V. L. S.: The Production of Wines (Fruit), *Fruit Products Jour.*, **16**: 106, 140 (1936).

COUCHE, D. D.: "Modern Detection and Treatment of Wine Diseases and Defects," Published in London by the author, The Avi Publishing Co., Inc. (representative), New York.

COWAN, M. R.: Musts, Wine and Metals, *Fruit Products Jour.*, **18**: 106 (1938).

COX, H. E.: "Chemical Analysis of Foods," Chap. VII, J. & A. Churchill, Ltd., London, 1938.

CRUESS, W. V.: "The Principles and Practice of Wine Making," 2d ed., The Avi Publishing Co., Inc., New York, 1947.

———: Preparation of Yeast Starters for Wine Making, *Fruit Products Jour.*, **13**: 166 (1934).

———: The Fermentation of White Wine, *Fruit Products Jour.*, **13**: 230 (1934).

———: Pasteurizing Wines of Low Alcohol Content, *Fruit Products Jour.*, **14**: 7 (1934).

———: The Tourne Disease of Wine, *Fruit Products Jour.*, **14**: 198 (1935).

———: Pasteurization of Wines, *Fruit Products Jour.*, **15**: 40 (1935).

———: Preservation of Bottled Sauterne Wines, *Fruit Products Jour.*, **15**: 228 (1936).

———: Wine in the Diet, *Fruit Products Jour.*, **15**: 260 (1936).

———: Further Data on the Effect of SO_2 in Preventing High Volatile Acidity in Wines, *Fruit Products Jour.*, **15**: 291, 324 (1936).

———: Tests on Unfortified Sweet Wines, *Wine Rev.*, **4**: 20 (February, 1936).

———: Checking Fermentation Temperatures, *Wine Rev.*, **4**: 16 (April, 1936).

———: Observations of '36 Season on Volatile Acid Formation in Muscat Fermentations, *Fruit Products Jour.*, **16**: 198 (1937).

———, E. M. BROWN, and F. C. FLOSSFEDER: Sweet Wines of High Alcohol Content, *Jour. Ind. Eng. Chem.*, **12**: 1277 (1916).

——— and L. HOHL: A Method of Improving Sweet Wines, *Fruit Products Jour.*, **16**: 261 (1937).

———, M. A. JOSLYN, and L. G. SAYWELL: "Laboratory Examination of Wines and Other Fermented Fruit Products," The Avi Publishing Co., Inc., New York, 1934.

———, T. Scott, H. B. Smith, and L. M. Cash: A Comparison of Various Treatments of Cement and Steel Wine-tank Surfaces, *Food Research*, **2**: 385–396 (1937).
———, C. Weast, and R. Gilliland: Summary of Practical Investigations on Film Yeast, *Fruit Products Jour.*, **17**: 229 (1938).
Curtis, M. J.: Bacterial Diseases of Wine with Reference to *Lactobacillus* and Mannitic Organisms, *Fruit Products Jour.*, **16**: 174 (1937).
Douglas, H. C., and W. V. Cruess: A *Lactobacillus* from California Wine: *Lactobacillus Hilgardii*, *Food Research*, **1**: 113 (1936).
——— and L. S. McClung: Characteristics of an Organism Causing Spoilage in Fortified Sweet Wines, *Food Rrsearch*, **2**: 471–476 (1937).
Dujardin, J.: "Notice sur les instruments de precision, appliqués a l'oenologie," J. Dujardin, 24 rue Pavée, Paris.
Eoff, J. R., H. Butler, and W. Melchior: Some Unusual Alcoholic Fermentations, *Ind. Eng. Chem.*, **12**: 1277 (1927).
Fabre, J. H., and E. Bremond: Arsenic in Grape Musts and Wines, *Ann. Fals.*, **31**: 149 (1938).
Goresline, H. E.: The Microbiology of Wine-Making, *Fruit Products Jour.*, **15**: 196 (1936).
——— and R. Wellington: New York Dry Red Wines, *Wines and Vines*, **17**: (No. 2) 6 (1936).
——— and ———: New York Champagnes, *Wines and Vines*, **17**: (No. 12) 5 (1936).
Guittonneau, L., et P. Pacottet: "Vins de Champagne et vins Mousseux," 2d ed., J. C. Baillière et fils, Paris, 1930.
Hedrick, U. P.: "Manual of North American Grapes," State of New York, Albany.
Herstein, K. M., and T. C. Gregory: "Chemistry and Technology of Wines and Liquors," D. Van Nostrand Company, Inc., 1935.
Hewitt, J. T.: "Chemistry of Wine Making," H. M. Stationery Office, London, 1928.
Hohl, L.: Further Observations on Production of Alcohol by *Saccharomyces ellipsoideus* in Syruped Fermentations, *Food Research*, **3**: 453 (1938).
——— and W. V. Cruess: Effect of Temperature, Variety of Juice, and Method of Increasing Sugar Content on Maximum Alcohol Production by *Saccharomyces Ellipsoideus*, *Food Research*, **1**: 405 (1936).
Husmann, G. C.: Grape Districts and Varieties in the United States, *U.S. Dept. Agr., Farmers' Bull.* 1689.
Joslyn, M. A.: The Possibilities and Limitations of the Artificial Aging of Wine, *Fruit Products Jour.*, **13**: 208 (1934).
———: The Composition of Wine and Must, *Fruit Products Jour.*, **13**: 326 (1934).
———: Preliminary Observations on the Mellowing and Stabilization of Wine, *Fruit Products Jour.*, **15**: 10 (1935).
———: Report on Volatile Acids in Wine, *Jour. Assoc. Official Agr. Chem.*, **21**: 166 (1938).
———: Determination of Acetaldehyde in Wines, *Ind. Eng. Chem.*, **10**: 364 (1938).
——— and M. A. Amerine: Commercial Production of Dessert Wines, *Univ. Calif., Bull.* 651, September, 1941.
———, and W. V. Cruess: Elements of Wine Making, *Calif. Agr. Ext. Ser., Circ.* 88, November, 1934.
——— and R. Dunn: Acid Metabolism of Wine Yeast. 1. The Relation of Volatile Acid Formation to Alcoholic Fermentation, *Jour. Am. Chem. Soc.*, **60**: 1137 (1938).

——— and G. L. Marsh: Methods of Wine Analysis, *Jour. Assoc. Official Agr. Chem.*, May, 1935.

Kayser, E.: The Races of Yeast and Their Significance in the Bouquet of Wine, *Chimie & industrie*, Spec. No. 619–625, May, 1924.

Kroemer, K., und G. Krumbholz: "Obst- und Beerenweine," Bruno Hempel, Braunschweig, Germany, 1932.

Lincoln, H. L.: Controlled Temperature Makes Better Wines, *Ice and Refrigeration*, **90:** 111 (April, 1936).

Loesecke, H. W. von, H. H. Mottern, and G. N. Pulley: Wines, Brandies and Cordials from Citrus Fruits, *Ind. Eng. Chem.*, **28:** 1224 (1936).

Marsh, G. L.: The Peculiarities of Carbon Dioxide in Wine, *Wine Rev.*, **4:** 17 (1936).

——— and M. A. Joslyn: Precipitation Rate of Cream of Tartar from Wine, *Ind. Eng. Chem.*, **27:** 1252 (1935).

Morris, M. M.: Volatile Acids of Wine, *Ind. Eng. Chem.*, **27:** 1250 (1935).

Mrak, E. M., L. Cash, and D. C. Caudron: Corrosion of Metals by Musts and Wines, *Food Research*, **2:** 439–454 (1937).

———, ———, and ———: Effects of Certain Metals and Alloys on Claret- and Sauterne-Type Wines Made from Vinifera Grapes, *Food Research*, **2:** 539 (1937).

——— and J. F. Fessler: Changes in Iron Content of Musts and Wines during Vinification, *Food Research*, **3:** 307 (1938).

Müller-Thurgau, H., und A. Osterwalder: Die Bakterien im Wein und Obstwein und die dadurch verursachten Veränderungen, *Centr. Bakt. Parasitenk.*, II *Abt.*, **36:** 129 (1913).

——— und ———: Weitere Beitrage zur Kenntniss der Mannitbakterien im Wein. *Centr. Bakt. Parasitenk.*, II *Abt.*, **48:** 1 (1917).

——— und ———: Über die durch Bakterien verursachte Zersetzung von Weinsäure und Glyzerin im Wein, *Ber. Schweiz. Versuchsanstaldt, Wadenswil*, **103** (1922).

National Fruit and Cider Institute. The Production of Fruit Wines, *Fruit Products Jour.*, **16:** 140 (1937).

Nelson, E. K.: Flavor of Alcoholic Beverages, *Food Research*, **2:** 221 (1937).

Niehaus, C. J.: The Principal South African Wine Diseases, *Farming S. Africa*, **4:** 475 (1930).

———: Mannitic Bacteria in South African Sweet Wines, *Farming S. Africa*, **6:** 443 (1932).

Ottavi, O.: "Enologia, Teoretico-Practico," 12th ed., Fratelli Ottavi, Casale Monferrato, Italy, 1931.

Pacottet, P.: "Vinification," J. C. Baillière et fils, Paris, 1926.

Pederson, C. S., H. E. Goresline, and E. A. Beavens: Pasteurization of New York State Wines, *Ind. Eng. Chem.*, **27:** 1257 (1935).

Quaccia, L.: The Semichon Process of Fermentation, *Fruit Products Jour.*, **14:** 169 (1935).

Saywell, L. G.: Clarification of Vinegar, *Ind. Eng. Chem.*, **26:** 379 (1934).

———: Effect of Filter Aids and Filter Materials on Wine Composition, *Ind. Eng. Chem.*, **27:** 1245 (1935).

Schanderl, H.: Kellerwirtschaftliche Fragen zur Schaumweinbereitung, *Wein und Rebe*, **20:** 10 (1938).

Searle, H. E., F. L. LaQue, and R. H. Dohrow: Metals and Wines, *Ind. Eng. Chem.*, **26:** 617 (1934).

Semichon, L.: Preparation of Wine by Continuous Fermentation, *Compt. rend.*, **176:** 1017 (1923).

SHAND, M. P.: "Book of Wines," Coward-McCann, Inc., New York, 1926.
SIMON, A. L.: "Wine and the Wine Trade," 2d ed., Sir Isaac Pitman & Sons, Ltd., London, 1934.
STOLL, H. F.: California Dry White Wines, *Wine and Vines*, **17**: (No. 3) 3 (1936).
TURBOVSKY, M. W., F. FILIPELLO, W. V. CRUESS, and P. ESAU: Observations on the Use of Tannin in Wine Making, *Fruit Products Jour.*, **14**: 106 (1934).
U. S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 1. Relating to the Issuance, Revocation, Suspension, and Amendment of Basic Permits, October, 1935, 1935.
U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 2. Relating to Non-industrial Use of Distilled Spirits and Wine, December, 1935, 1936.
U. S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 4. Relating to Labeling and Advertising of Wine, December, 1935, 1936.
U.S. Dept. of the Treasury, Federal Alcohol Administration. Federal Alcohol Administration Act as in Effect on May 15, 1937, 1937.
VAN LAER, M. H.: "La chimie des fermentations," Masson et Cie, Paris, 1935.
VERTRE, J.: "Traité de vinification. Pratique et rationnelle," 3 vols., Librarie Coulet. A. Dubois et R. Poulain, Montpellier, France, 1930–1931.
WAGNER, P. M.: "American Wines and How to Make Them," Alfred A. Knopf, Inc., New York, 1933.
WILEY, H. W.: American Wines at the Paris Exposition of 1900: Their Chemical Compositions and Character, *U.S. Dept. Agr., Bur. Chem., Bull.* 72, 1903.
———: "Beverages and Their Adulteration," P. Blakiston's Son & Company, Philadelphia, 1919.
WILLIAMS, A.: Plate Heat Exchangers for the Processing of Wines, *Fruit Products Jour.*, **18**: 294 (1939).
WINKLER, A. J.: Making Red Wines in Algeria, *Wine Rev.* (July, 1935).
———: Temperature and Varietal Interrelations in Central-western Europe and Algeria, *Wines and Vines* (February, 1936).
——— and M. A. AMERINE: What Climate Does, *Wine Rev.* (June, July, 1937).
——— and ———: Color in California Wines. I. Methods for Measurement of Color, *Food Research*, **3**: 429 (1938); II. Preliminary Comparisons of Certain Factors Influencing Color, *Food Research*, **3**: 439 (1938).
WINTON, A. L., and K. B. WINTON: "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.
WOODMAN, A. G.: "Food Analysis," 4th ed., McGraw-Hill Book Company, Inc., 1941.

Some Patents

BUCK, H. G., and A. W. ASPEN: Wine Dispensing Rack, U.S. Patent 2,060,170, Nov. 10, 1936.
FOWLE, G. W.: Wine Press, U.S. Patent 2,096,097, Oct. 19, 1937.
KREBS, E. T.: Method of Aging Wine, U.S. Patent 2,080,138, May 11, 1937.
SAYWELL, L. G.: Wine and Vinegar Clarification Process, U.S. Patent 2,043,713, June 9, 1936.
SCOTT, A. B., and G. R. ROBERTSON: Wine Preserving Apparatus, U.S. Patent 2,036,505, Apr. 7, 1936.
TRESSLER, D. K.: Wine Process, U.S. Patents 2,181,838, 2,181,839, Nov. 28, 1939.
VINGERHOETS, G.: Producing a Wine, U.S. Patent 1,957,375, May 1, 1934.
WILLIAMSON, W. W.: Wine Cooling Apparatus, U.S. Patent 2,120,305, June 14, 1938.

CHAPTER VIII

THE DISTILLING INDUSTRIES

The distilling industries are those concerned with the production of distilled spirits: rum, whiskey, brandy, gin, and cordials and liqueurs.

Production Statistics.—Table 46 shows the production of distilled spirits for the fiscal year 1946, ended June 30, 1946.

TABLE 46.—PRODUCTION OF DISTILLED SPIRITS, BY KINDS AND BY STATES, FISCAL YEAR 1946[1,2]
(Tax gallons)

State	Whiskey	Rum	Gin	Brandy	Other spirits	Total
Arkansas	.....	.....	.....	770	.....	770
California	379,607	.....	313,768	33,145,469	1,520,191	35,359,035
Colorado	36,410	.....	.....	.....	.....	36,410
Connecticut	46,824	.....	1,637	3,746	52,502	104,709
Florida	.....	.....	.....	472,159	.....	472,159
Georgia	172,971	.....	.....	37,322	105,164	315,457
Idaho	.....	.....	.....	.....	622,306	622,306
Illinois	15,969,588	.....	3,025,709	.....	19,311,498	38,306,795
Indiana	15,740,714	.....	1,448,521	.....	20,462,347	37,651,582
Iowa	292,586	.....	.....	.....	15,471	308,057
Kentucky	63,459,394	1,141,301	199,920	.....	20,456,577	85,257,192
Louisiana	.....	66,535	.....	.....	365,691	432,226
Maine	.....	.....	.....	8,554	.....	8,554
Maryland	9,815,294	.....	1,138,851	.....	12,168,851	23,122,996
Massachusetts	1,796,681	1,133,203	.....	.....	3,099,236	6,029,120
Michigan	.....	.....	.....	.....	7,152,933	7,152,933
Minnesota	1,598	.....	.....	.....	18,737	20,335
Missouri	1,258,688	.....	42,048	.....	560,821	1,861,557
New Hampshire	451,209	.....	8,844	.....	753,224	1,213,277
New Jersey	.....	.....	.....	140,062	.....	140,062
New York	.....	.....	1,081,945	51,732	4,943,095	6,076,772
Ohio	1,235,169	.....	.....	60,142	7,016,159	8,311,470
Oregon	.....	.....	.....	223,340	.....	223,340
Pennsylvania	35,340,916	316,541	236,159	13,918	13,214,747	49,122,281
South Dakota	132,370	.....	.....	.....	250,977	383,347
Tennessee	117,591	.....	.....	15,810	15,881	149,282
Vermont	21,317	.....	.....	.....	379,125	400,442
Virginia	1,085,653	.....	.....	30,267	322,079	1,437,999
Washington	.....	.....	.....	213,094	.....	213,094
Wisconsin	109,936	.....	.....	.....	223,142	333,078
Total	147,464,516	2,657,580*	7,497,402	34,416,385†	113,030,754‡	305,066,637

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1946

[2] Production of whiskey, rum, gin, and spirits by registered distilleries, and brandy and spirits by fruit distilleries.

* Includes 1,551,303 tax gal. removed for denaturation.

† Includes 7,005,916 tax gal. of spirits-fruit.

‡ Production for beverage and industrial purposes. For industrial purposes 2,934,875 tax gal. were produced during July; 24,961,923 tax gal. during August; and 2,021,823 tax gal. during September, 1945.

An examination of this table shows that Kentucky, Pennsylvania, Illinois, Indiana, and Maryland led the country in the production of whiskey (in the order named), while rum was produced principally in Kentucky and Massachusetts.

Illinois led the United States in the production of gin, with Indiana, Maryland, and New York following in the order named.

California was outstandingly the leader in brandy manufacture, while Indiana led in the production of other spirits.

RUM

"Rum" is any alcoholic distillate from the fermented juice of sugarcane, sugarcane sirup, sugarcane molasses, or other sugarcane by-products distilled at less than 190° proof (whether or not such proof is further reduced prior to bottling to not less than 80° proof) in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to rum; and includes mixtures solely of such distillates.[1]

Rum is manufactured in general in those countries which grow sugarcane or import molasses or other sugarcane products. It is made principally in the United States, Puerto Rico, Cuba, Jamaica, St. Thomas and St. Croix (Virgin Islands of the United States), British Virgin Islands, Demerara, Barbados, Uruguay, Martinique, Trinidad, Haiti, Santo Domingo, the Leeward Islands, Guadelupe, Mexico, Grenada, and Antigua.

Composition.—Table 47 gives analyses of some rums from different sources.

Acids (volatile and nonvolatile), esters, fusel oil, and aldehydes are regular constituents of rum. Ethyl acetate is the most common ester produced, while both acetaldehyde and furfural ($C_4H_3O \cdot CHO$) are always found, in traces at least, in rum.

Types.—Rums made in the United States, with certain exceptions, may be classified as heavy-bodied rums.[2] They contain only the congenerics (acids, esters, etc.) that are accumulated during distillation and aging in charred white oak barrels. Most of the rums are distilled at a proof less than 160° and aged for several years. They contain no coloring matter added by the distillers.

Rums produced outside the United States, with exceptions, vary from very light bodied to very heavy bodied. Cuba produces some of lightest bodied rums, while Jamaica manufactures the heaviest bodied rums outside the United States. Substances are frequently added to

[1] Dept. of the U.S. Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

[2] Valaer, P., Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

TABLE 47.—ANALYSES OF SOME RUMS[1]
(Grams per 100 liters not calculated to proof)

Proof	pH	Total acid (as acetic)	Volatile acid (as acetic)	Esters (as ethyl acetate)	Fusel oil (as amyl alcohol)	Solids	Aldehydes	Furfural	Color in 0.5-in. cell	Source, comments
103.0	4.76	9.6	9.6	16.7	123.2	8	4.4	0.0	0.2	Pennsylvania, new
103.6	4.54	33.6	26.4	23.8	124.3	48	4.7	Trace	4.0	Pennsylvania, 0.5 year old
104.8	4.52	36.0	28.8	26.4	124.0	50	5.5	0.8	4.5	Pennsylvania, 1 year old
107.8	4.44	50.4	40.8	33.4	132.0	94	6.8	0.8	7.0	Pennsylvania, 2 years old
102.0	4.28	14.4	14.4	43.1	102.1	16	40	5.5	0.0	Kentucky, new
104.4	4.17	80.4	67.2	60.7	108.6	168	40	8.2	13.5	Kentucky, 1 year old
106.8	4.28	88.8	73.2	73.0	116.2	204	34.3	12.0	17.0	Kentucky, 2 years old
100.6	4.68	38.4	36.0	22.0	91.7	6	4.0	1.2	0.0	Massachusetts, new
100.4	4.42	84.0	74.0	29.0	91.5	86	6.4	1.2	7.5	Massachusetts, 0.5 year old
101.4	4.37	93.4	86.4	45.5	93.3	142	7.2	1.4	11.0	Massachusetts, 1.5 years old
102.4	4.30	98.4	86.4	51.0	96.8	162	7.2	1.6	13.0	Massachusetts, 2 years old
135.2	4.07	184.8	148.8	216.0	450.6	274	48	7.2	18.0	Old New England, approx. 19 years old
87.3	5.03	13.2	12.0	11.4	49.3	678	1.6	0.1	14.5, caramel	Cuba, Ron Bacardi Superior, gold
92.8	8.05	4.8	2.4	44.0	59.8	205	7.0	0.8	15.5	St. Croix cane juices, 2 years old
98.6	4.17	57.6	48.0	59.8	91.5	450	20.0	4.0	23.0, caramel	Jamaica, 15 years old
92.8	4.32	62.4	43.2	26.4	68.6	1,510	0.8	0.0	34.0, caramel	Demerara

[1] VALAER, P., Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

such rums to bring about the desired characteristics, one of these substances being caramel for artificial color (not all countries permit these additions, however).

Table 48 gives the analyses of some light-, medium-, and heavy-bodied rums.

Production of Rum. *Fermentation.*—The process for the manufacture of rum is similar in many respects to that in which industrial alcohol is produced. Methods for the propagation of pure cultures of yeast, the building up of a starter, the reaction of the main mash, the concentration of the fermentable sugar, the temperature of the fermentation, and even the principal end products are similar. Some rums (Jamaica) are made by spontaneous fermentation, however.

In the manufacture of rum, the careful selection of the raw materials, the control of the fermentation, proper distillation, and the aging of the distilled product are essential for the production of a fine rum.

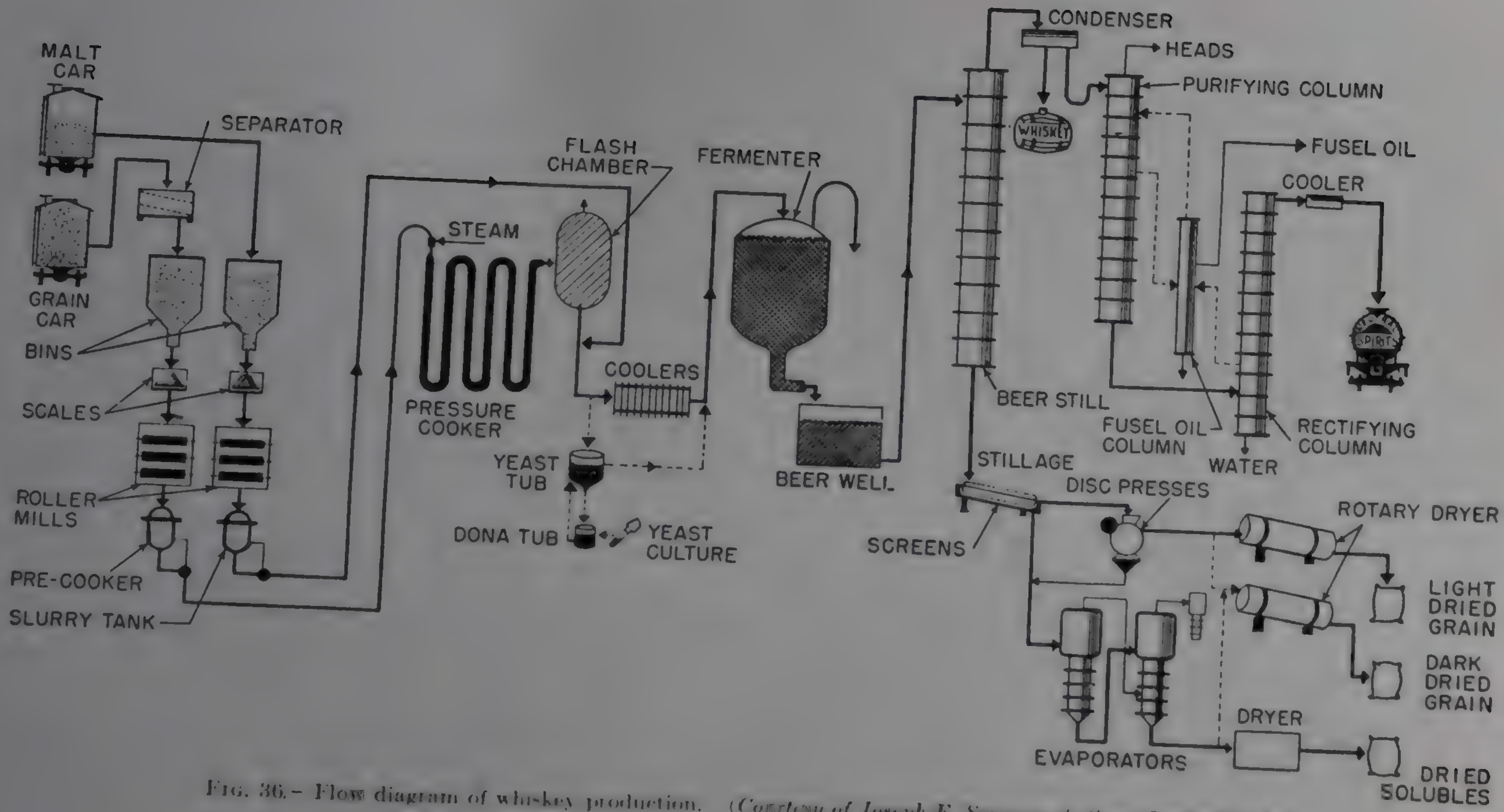


FIG. 36.– Flow diagram of whiskey production. *(Courtesy of Joseph E. Seagram & Sons, Louisville, Ky.)*

Strains of *Saccharomyces cerevisiae* or of other yeasts, such as species of *Schizosaccharomyces*, may be used to pitch (inoculate) the mash. Great care must be exercised to maintain the selected yeast in pure culture, if the fermentation is to be successful.

As already indicated, rum is prepared from sugar-cane products. In the United States only blackstrap molasses is used in rum manufacture. This substance can be purchased at relatively low cost when procured in large quantities. It is a product of the sugar mill after the crystallizable sugar has been removed, and usually contains 40 to 55 per cent of fermentable sugar.

TABLE 48.—ANALYSES OF SOME LIGHT-, MEDIUM-, AND HEAVY-BODIED RUMS[1]

Type of rum	Manufacture and location	Grams per 100 liters of 100 proof								
		Proof	Total acid (as acetic)	Esters (as ethyl acetate)	Aldehyde	Furfural	Fusel oil (as amyl alcohol)	Color	Wood extract	
Light bodied	Bacardí (Cuba), Carta Blanca[2]	89.6	9.1	11.7	5.4	0.1	65.0	Light	Slight	Bottled for sale in the United States
	Castillo (Cuba), Carta Oriente[3]	87.8	9.7	7.0	7.3	0.1	79.0	Dark	Slight	Bottled for sale in the United States
Medium bodied	Ron Rico (Puerto Rico), White Label[4]	82.9	28.	32.	19.	0.6	136.	Light	Present	Bottled for sale in the United States
	Bacardí (Cuba), "Extra Superior, 1873"[5]	90.1	53.	25.	11.	0.8	80.	Dark	Present	Bottled for sale in the United States
Heavy bodied	Wray's 3 Dagger (Jamaica)[6]	99.8	34.0	56.0	18.0	4.0	238.	Dark	Present	For the United States trade. Bottled sample
	(Jamaica) for sale to Germany[7]	149.	141.	565.	19.0	5.4	114.	Dark	Present	High-proof. From a barreled sample

[1] Courtesy of Dr. Walter C. Tobie.

[2] Typical of a very light-flavored Cuban rum, such as is frequently used for highballs and cocktails.

[3] Although the color is dark, the flavor of this rum is light.

[4] An early sample (about 1934) for shipment to the United States.

[5] This is one of the heaviest Cuban rums. The flavor is relatively rich and fruity.

[6] A typical Jamaica rum for sale in the United States. The flavor is high.

[7] Bulk rum, said to have been prepared in Jamaica for the German trade. Note the excessively high ester value.

A concentration of molasses that contains from 12 to 14 per cent of fermentable sugars may be used in preparing the main mashes. Ammonium sulphate, and occasionally phosphates, may be added as a source of food for the yeast. Dunder, *i.e.*, distillation slops which may be decomposed, may be used to give the rum a heavier flavor and to supply nutrient material.

Sulphuric acid is used to adjust the pH of the mash to 4.0 to 4.7, the latter pH favoring a rapid fermentation and the production of a light-flavored rum. The use of lower pH values favors slower fermentations and the production of heavy-bodied rums, such as the Jamaica type. It is usually necessary to add acid to the mash, unless it has been heat sterilized, to inhibit the development of undesirable bacteria.

The fermentation tanks must be cleaned carefully after being used if a clean fermentation and light-bodied rum are desired. Steam, sulphuric acid, ammonium bifluoride, or other agents may be used to prevent the development of undesirable flora in the tank.

A fermentation temperature of about 70°F. (initial) to 96°F. (final) is satisfactory. Low temperatures favor a slow fermentation, while at high temperatures volatile constituents are lost.

Although the main fermentation is nearly complete within 2 days, the mashes are permitted to ferment for 3 to 7 days before being distilled. A 6-day or 144-hr. fermentation period is used in at least one modern distillery in this country.

The fermented mash is sometimes designated as "beer."

Distillation.—Distillation is an important process in the production of rum. Mashes that are distilled at high proof produce light-flavored rums, which are expected to be consumed shortly. Only small changes take place in such products during storage, for the rums contain but small quantities of acids, esters, fusel oil, and other congeneric substances. As stated earlier, most rums in this country are distilled at a proof of less than 160°.

Various types of distillation equipment are used: pot stills, continuous-type stills with combined rectifying columns, beer stills with doublers, chambered stills, and other modifications.

In the United States, rum distilled at a low proof is usually adjusted to 100° proof before being aged.

Aging.—Aging is a process for improving the character of rum, for decreasing the undesirable flavors, and for greatly increasing the pleasant ones. Flavor, color, and aroma are developed, while the product is mellowed. During the first 6 months of the process, the main increases in esters, acids, solids, and color take place. In a freshly distilled rum, the quantity of esters usually exceeds that of the acids, but after storage

for less than 6 months the ratio changes. Indeed, the acid content is usually greater than the ester content after two years,[1] owing largely to the acidic products extracted from the wood of the barrel.

Rum is usually aged in charred white oak barrels, although some is aged in plain cooperage. The aging is permitted to proceed for a few months or for several years (in Government bonded warehouses in the United States).

Some rum is "quick-aged" by placing charred white oak chips in the rum before it is stored. Quick-aging may also be accomplished by circulating the rum over oak chips. Heat and aeration are sometimes used. Quick-aging processes are not particularly satisfactory, however.

Government Supervision.—The manufacture of rum in the United States and its possessions is carried out under Government supervision. The quantity of raw material used and rum produced are carefully measured. The Bureau of Internal Revenue is interested in taxes, and the Federal Alcohol Administration in the promulgation of regulations concerning standards for identification, such as labeling and advertising.

Labeling.—According to the Federal Alcohol Administration Division,[2] a bottled rum should contain the following information on the label: the class and type, the alcoholic content, the net contents, and the presence of "artificial or excessive coloring or flavoring." A statement concerning the age of the rum may also be placed on the label, if desired.

Uses.—Rum may be used in the preparation of ice cream, candies, and mincemeat; in the curing of tobacco; as a beverage; and as a medicinal.

Definitions and Regulations.—"New England rum" is rum that is produced in the United States, is distilled at less than 160° proof and is a straight rum and not a mixture of rums.[3]

Puerto Rico, Cuba, Demerara, Barbados, St. Croix, St. Thomas, Virgin Islands, Jamaica, Martinique, Trinidad, Haiti, and San Domingo rum are not distinctive types of rum. Such names are not generic but retain their geographic significance. They may not be applied to rum produced in any other place than the particular region indicated in the name, and may not be used as a designation of a product as rum, unless such product is rum as defined [see page 223].

Imitation rum—(1) neutral spirits or other distilled spirits which have been added thereto or which contain synthetic or imitation rum flavoring materials, and (2) rum which has added thereto neutral spirits or other distilled spirits than rum are "imitation rum" and shall be so designated.

[1] VALAER, *loc. cit.*

[2] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

[3] *Ibid.*

WHISKEY

According to Article II, Section 21, Class 2 of the Federal Alcohol Administration Act, Regulations 5,[1]

"Whisky" is an alcohol distillate from a fermented mash of grain distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to whisky, and withdrawn from the cistern room of the distillery at not more than 110° proof and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof; and also includes mixtures of the foregoing distillates for which no specific standards of identity are prescribed herein. . .

Types.—There are several types of whiskey. Standards for the identity of American types and some others are stated later in this chapter.

Composition of Whiskies.—Whiskies contain approximately 50 per cent by volume ethyl alcohol. The flavor and bouquet are due mainly to the impurities or congeneric substances that they contain, however. These accumulate during the production and storage of the whiskey.

In whiskey are found acids, esters, aldehydes, furfural, fusel oil, and solids, in addition to ethyl alcohol and water. The principal acids are acetic and valeric acids,[2] but traces of propionic and other acids are found also. The combination of ethyl alcohol with these acids yields ethyl acetate, ethyl valerate, and ethyl propionate, respectively, while the amyl alcohols present in the fusel oil form amyl acetate, amyl valerate, and other esters. Aldehydes include acetaldehyde and those resulting from the oxidation of small amounts of some of the higher alcohols during aging. Fusel oil is composed mainly of higher alcohols.

In Table 49 are given the average analyses for 31 different samples (barrels) of whiskey, of which 14 were rye whiskey, 13 being prepared by the sweet-mash and 1 by the sour-mash method, and of which 17 were bourbon whiskey, 13 being prepared by the sour-mash and 4 by the sweet-mash method. The average data for the rye and bourbon whiskies are also given. The effect of aging on the quantities of the various components of whiskey is illustrated. The results are expressed in grams per 100 liters, calculated to the original volume of the whiskey. The acids are calculated as acetic acid, the esters and aldehydes as acetic, the fusel oil as amyl alcohol.

Production of Whiskey.—The flavor, aroma, and characteristics of the final product depend on the nature of the raw materials and the fermentation, the method of distillation, and the aging process.

[1] *Ibid.*

[2] WOODMAN, A. G., "Food Analysis," 4th ed., McGraw-Hill Book Company, Inc., 1941.

1. *Raw Materials.*—Grains are the raw materials from which whiskies are produced, the usual ones being rye, corn, wheat, and barley.

In the United States, rye and bourbon are the principal types of whiskey manufactured. Rye whiskey is generally prepared from rye and rye malt or from rye and barley malt. A typical mash may contain 80 per cent rye and 20 per cent barley malt. At least 51 per cent rye is

TABLE 49.—AVERAGE ANALYSES OF SOME WHISKIES[1]
(Grams per 100 liters calculated to original volume)

Age, years	Type	Color	Solids	Acids	Esters	Aldehydes	Furfural	Fusel oil
New	Whole	....	20.0	6.4	15.0	4.03	0.71	96.8
	Rye	....	13.6	4.7	13.7	4.91	0.97	83.2
	Bourbon	....	26.0	7.7	17.2	3.26	0.44	108.6
1	Whole	7.3	101.5	37.8	29.9	7.08	1.5	106.2
	Rye	8.4	114.6	41.8	35.3	8.71	1.7	106.8
	Bourbon	6.4	90.1	34.4	24.9	5.55	1.3	105.8
2	Whole	8.6	124.2	46.1	42.9	8.34	1.7	108.1
	Rye	10.6	133.6	49.8	49.3	9.02	1.9	109.7
	Bourbon	6.7	114.8	42.7	37.3	7.78	1.4	107.3
3	Whole	10.2	140.2	51.1	48.4	9.47	1.8	106.3
	Rye	11.5	150.4	54.4	54.3	9.80	2.2	104.4
	Bourbon	8.3	130.7	47.8	42.5	9.15	1.5	107.3
4	Whole	10.2	140.4	51.6	50.9	10.2	1.9	104.3
	Rye	11.6	153.1	54.2	57.2	11.2	2.2	102.0
	Bourbon	8.9	127.7	48.9	45.0	9.3	1.5	106.3
5	Whole	11.1	149.2	52.2	51.1	10.2	1.9	100.4
	Rye	12.2	158.8	54.8	57.5	11.3	2.5	100.1
	Bourbon	10.0	140.2	49.8	45.0	9.2	1.5	100.7
6	Whole	11.1	151.4	53.2	50.7	10.2	1.9	104.7
	Rye	12.3	161.0	54.8	55.5	11.3	2.4	105.9
	Bourbon	10.1	142.5	51.8	45.2	9.1	1.4	103.8
7	Whole	11.1	154.0	52.2	51.1	9.8	1.8	99.9
	Rye	12.0	161.3	51.9	56.6	10.6	2.2	98.8
	Bourbon	10.2	147.1	52.4	46.4	9.0	1.4	101.6
8	Whole	10.5	155.2	53.1	50.9	9.6	1.8	98.0
	Rye	11.1	163.8	52.6	56.7	10.6	2.2	99.0
	Bourbon	10.0	147.7	53.6	45.9	8.8	1.5	97.1

[1] CRAMPTON, C. A., and L. M. TOLMAN, *Jour. Am. Chem. Soc.*, **30**: 98 (1908).

required by law. Bourbon, on the other hand, is prepared from corn (maize or Indian), barley malt or wheat malt, and usually another grain. A typical mash may contain, for example, 70 per cent corn, 15 per cent rye and 15 per cent malt, or 65 per cent corn, 23 per cent rye and 12 per cent malt. The mash must always contain at least 51 per cent corn (see the standards of identity for whiskey given in this section).

The preparation of a mash for whiskey is similar in fundamental details to the preparation of a mash in the brewing industry, except that the solids are not removed (Chap. VI). The enzymes of the malt convert the starches of the cooked grains to dextrins and sugars. At the same time, the protein molecules are degraded sufficiently to furnish the yeast with a satisfactory source of nitrogen.

2. *Fermentation.*—The resultant mash is fermented by a strain of distiller's yeast, *Saccharomyces cerevisiae*, by either the sweet- or sour-mash method. In the former method, which is commonly used, the mash is inoculated directly with the yeast. Less time is required for this fermentation, generally, and a higher yield of alcohol is obtained than from fermented sour mashes. In the latter method the spent slops and barm (yeast) from tanks previously set and fermented are mixed with the mash before fermentation.

Bacteria play a part in the production of esters in whiskey manufacture, the temperatures attained during fermentation favoring their growth. Not infrequently the distiller may grow his selected culture of yeast in a medium containing lactic acid bacteria, the lactic acid favoring the growth of yeast, inhibiting certain undesirable types of microorganisms, and contributing to the aroma, flavor, and characteristics of the whiskey.

3. *Distillation.*—Both the type of still and the method of distillation are important, for they contribute to the quality of the product. The continuous still, with doubler, and the three-chambered still, with doubler, are two commonly used stills.

4. *Aging.*—During the process of aging, definite changes take place in certain of the congeneric substances of whiskey as the result of storing it in oak containers. These changes have been investigated in great detail by Crampton and Tolman (1908) and by Valaer and Frazier (1936).

"Age" for "American type whiskeys, other than corn whiskey, straight corn whiskey, blended corn whiskey, and blends of straight corn whiskey, produced on or after July 1, 1936, means the period during which the whiskey has been kept in charred new oak containers."[1]

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, as amended to Mar. 1, 1939.

Whiskey is stored in heated or unheated warehouses for aging, heat causing greater extraction of substances from the wood of the container.

CHANGES DURING AGING.- -During aging in white oak barrels, changes take place in the solids, esters, acids, fusel oil, aldehydes, furfural, and color, the largest increase in solids, esters, acids, and color occurring during the first half year of storage.[1]

The solids of whiskey, known also as extract, are derived from the wood of the container. During the first 6 months of storage, Valaer and Frazier found an average increase of about 70 g. of solids per 100 liters. The quantity of extract was progressively smaller during each succeeding 6-month period, being apparently 4 g. per 100 liters during the period between the forty-second and forty-eighth months of storage.

Since there is very little change in the ester content of a whiskey during quick-aging, the determination of esters may be used as a dependable index of the age of the whiskey.

The acids of whiskey are both volatile and fixed acids. The larger part of the increase in total acids during storage is due to volatile acids. Some fixed acids are extracted from the wood of the container.

During storage in charred oak barrels, there is an actual loss in the quantity of fusel oil, according to Valaer and Frazier. The fusel-oil determination gives information concerning the method of distillation and is, therefore, considered to be an important determination.

There is an actual loss of aldehydes during storage.

Some furfural is extracted from charred oak barrels during the first 6 months of storage, but very little appears later. Uncharred barrels do not contribute furfural.

QUICK-AGING.—The process of quick-aging of whiskey is used extensively, especially for whiskies that are to be marketed shortly after manufacture. Quick-aging produces but little change in the ester content and no effect on the fusel oil. It increases the aldehydes only slightly. Solids, acids (mainly the nonvolatile ones), furfural, and color are increased. Color depth is readily varied by means of this process. The following table illustrates the effect of quick-aging on a whiskey.

Heat, charred or uncharred wood chips, and charred barrels are used in some of the quick-aging processes.

CHANGES IN GLASS.—Although extensive changes do not usually occur in whiskies stored in glass containers, evidence indicates that there may be a slight decrease in acids in the case of some whiskies, especially those which are not new. Valaer and Frazier suggest that this reduction in acidity may be due to the alkali dissolved from the glass, to the establish-

[1] VALAER, P., and W. H. FRAZIER, *Ind. Eng. Chem.*, **28**: 92 (1936).

TABLE 50.—DIFFERENCE IN SAME WHISKY WITH AND WITHOUT QUICK-AGING[1]

	Grams per 100 liters calculated to proof	
	Quick-aged	Not quick-aged
Total acids	11.9	5.9
Esters	14.3	15.6
Fusel oil	124 3	127.6
Solids	33.6	11.2
Color	3.5	0.0
Furfural	1.0	0.0

[1] VALAER, P., and W. H. FRAZIER, *Ind. Eng. Chem.*, **28**: 92 (1936).

ment of a change in equilibrium, or to an oxidation change. Esters tend to increase as does the color, while furfural is reduced in quantity.

Federal Control.—The manufacture of whiskey is carried out under Government supervision from the weighing of the grain to the sale of the whiskey. The Bureau of Internal Revenue and the Federal Alcohol Administration Division are much interested in the control of the whiskey production. For details in connection with importing, bottling, labeling, and advertising, the reader is referred to the publications of the latter division, some of which are listed in the references given at the end of this section.

Standards of Identity.—Standards of identity for different types of whiskies, under the provisions of the Federal Alcohol Administration Act, are as follows ("American type" whiskies being specified in subsection (*a*) through (*j*)):

(*a*) "Rye whiskey," "bourbon whiskey," "wheat whiskey," "malt whiskey," or "rye malt whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 51% rye grain, corn grain, wheat grain, malted barley grain or malted rye grain, respectively, and, if produced on or after March 1, 1938, stored in charred new oak containers, and also includes mixtures of such whiskeys where the mixture consists exclusively of whiskeys of the same type. "Corn whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 80% corn grain, stored in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood, and also includes mixtures of such whiskey.

(*b*) "Straight whiskey" is an alcoholic distillate from a fermented mash of grain distilled at not exceeding 160° proof and withdrawn from the cistern room of the distillery at not more than 110° and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof, and is—

(1) Aged for not less than twelve calendar months if bottled on or after July 1, 1936, and before July 1, 1937; or

(2) Aged for not less than eighteen calendar months if bottled on or after July 1, 1937, and before July 1, 1938; or

(3) Aged for not less than twenty-four calendar months if bottled on or after July 1, 1938.

The term "straight whiskey" also includes mixtures of straight whiskey which, by reason of being homogeneous, are not subject to the rectification tax under the Internal Revenue Laws.

(*c*) "Straight rye whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is rye grain.

(*d*) (1) "Straight bourbon whiskey" is straight whiskey distilled[1] from a fermented mash of grain of which not less than 51% is corn grain.

(3) "Straight corn whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 80% is corn grain, aged for the required period in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood.

(*e*) "Straight wheat whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is wheat grain.

(*f*) "Straight malt whiskey" and "straight rye malt whiskey" are straight whiskey distilled from a fermented mash of grain of which not less than 51% of the grain is malted barley or malted rye, respectively.

(*g*) "Blended whiskey" (whiskey—a blend) is a mixture which contains at least 20% by volume of 100° proof straight whiskey and, separately or in combination, whiskey or neutral spirits, if such mixture at the time of bottling is not less than 80° proof.

(*h*) "Blended rye whiskey" (rye whiskey—a blend), "blended bourbon whiskey" (bourbon whiskey—a blend), "blended corn whiskey" (corn whiskey—a blend), "blended wheat whiskey" (wheat whiskey—a blend), "blended malt whiskey" (malt whiskey—a blend) or "blended rye malt whiskey" (rye malt whiskey—a blend) is blended whiskey which contains not less than 51% by volume of straight rye whiskey, straight bourbon whiskey, straight corn whiskey, straight wheat whiskey, straight malt whiskey, or straight rye malt whiskey, respectively.

(*i*) "A blend of straight whiskeys" (blended straight whiskeys), "a blend of straight rye whiskeys" (blended straight rye whiskeys), "a blend of straight bourbon whiskeys" (blended straight bourbon whiskeys), "a blend of straight corn whiskeys" (blended straight corn whiskeys), "a blend of straight wheat whiskeys" (blended straight wheat whiskeys), "a blend of straight malt whiskeys" (blended straight malt whiskeys), and "a blend of straight rye malt whiskeys" (blended straight rye malt whiskeys) are mixtures of only straight whiskeys, straight rye whiskeys, straight bourbon whiskeys, straight corn whis-

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Amendment 5 to Regulations 5.

keys, straight wheat whiskeys, straight malt whiskeys, or straight rye malt whiskeys, respectively.

(*j*) "Spirit whiskey" is a mixture (1) of neutral spirits and not less than 5% by volume of whiskey, or (2) of neutral spirits and less than 20% by volume of straight whiskey, but not less than 5% by volume of straight whiskey, or of straight whiskey and whiskey, if the resulting product at the time of bottling be not less than 80° proof.

(*k*) "Scotch whiskey" is a distinctive product of Scotland, manufactured in Scotland in compliance with the laws of Great Britain regulating the manufacture of Scotch whiskey for consumption in Great Britain, and containing no distilled spirits less than three years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such mixture is "blended Scotch whiskey" (Scotch whiskey—a blend). "Scotch whiskey" shall not be designated as "straight."

(*l*) "Irish whiskey" is a distinctive product of Ireland, manufactured either in the Irish Free State or in Northern Ireland, in compliance with the laws of those respective territories regulating the manufacture of Irish whiskey for consumption in such territories, and containing no distilled spirits less than three years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Irish whiskey" (Irish whiskey—a blend). "Irish whiskey" shall not be designated as "straight."

(*m*) "Canadian whiskey" is a distinctive product of Canada, manufactured in Canada in compliance with the laws of the Dominion of Canada regulating the manufacture of whiskey for consumption in Canada, and containing no distilled spirits less than two years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Canadian whiskey" (Canadian whiskey—a blend). "Canadian whiskey" shall not be designated as "straight."

(*n*) "Blended Scotch type whiskey" (Scotch type whiskey—a blend) is a mixture made outside Great Britain and composed of—

(1) Not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at not more than 160° proof, from a fermented mash of malted barley dried over peat fire, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof, and

(2) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

(*o*) "Blended Irish type whiskey" (Irish type whiskey—a blend) is a product made outside Great Britain or the Irish Free State and composed of—

(1) A mixture of distilled spirits distilled in pot stills at not more than 171° proof, from a fermented mash of small cereal grains of which not less than 50% is dried malted barley, and unmalted barley, wheat, oats, or rye grains, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof; or

(2) A mixture consisting of not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at approximately 171° proof, from a

fermented mash of dried malted barley, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof; and

(3) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

BRANDIES

The following standards of identity for brandies are quoted from Regulations 5 (Article II, Sec. 21, Class 4) of the Federal Alcohol Administration.[1]

(*a*) "Brandy" is a distillate, or a mixture of distillates, obtained solely from the fermented juice or mash of fruit (1) distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to brandy; and (2) bottled at not less than 80° proof: and shall also include such distillates, aged for a period of not less than fifty years, and bottled at not less than 72° proof, in cases where the reduction in proof below 80° is due solely to losses resulting from natural causes during the period of aging.

(*b*) "Brandy," without appropriate qualifying words, or "grape brandy," is the distillate obtained from grape wine or wines under the conditions set forth in subsection (*a*) of this class, and includes mixtures solely of such distillates.

(*c*) "Apple brandy" (apple jack), "peach brandy," "cherry brandy," "apricot brandy," "orange brandy," "raisin brandy," and other fruit brandies are distillates obtained from the fermented juice or mash of the respective fresh or dried or otherwise treated fruits under the conditions set forth in subsection (*a*) of this class, and include mixtures composed wholly of one kind of such distillates. The designation shall contain the name of the fruit used, and, if other than whole fresh fruit is used, the word "dried" or such other term as may be appropriate. Brandy derived from raisins shall be designated as "raisin brandy."

(*d*) "Cognac" or "Cognac brandy" is grape brandy distilled in the Cognac Region of France, which is entitled to be designated as "Cognac" by the laws and regulations of the French Government; and includes mixtures of such brandy.

Brandies usually contain 40 to 50 per cent ethyl alcohol by volume, but may sometimes contain as little as 36 per cent alcohol by volume [paragraph (*a*) above].

A large amount of the wine manufactured in California (more than one-half, according to Goresline),[2] is distilled to produce brandy. Approximately 75 per cent of such brandy is used to fortify wines.

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

[2] GORESLINE, H. E., Notes on Wine Manufacture in the United States, *U.S. Dept. Agr.* (mimeographed sheets), 1936.

GINS

(a) "Distilled gin" is a distillate obtained by original distillation from mash, or by the redistillation of [illegible] spirits, over or with juniper berries and other aromatics customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and bottled at time of bottling to not less than 80° proof, and includes mixtures solely of such distillates.

(b) "Compounded gin" is the product obtained by mixing neutral spirits with distilled gin or gins or other flavoring materials customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and bottled at time of bottling to not less than 80° proof, and includes mixtures solely of such distillates.

(c) "Dry gin," "London dry gin," "Holland gin," "Geneva gin," "Old Tom gin," "Tom gin," and "London gin" are the types of gin known under such designations, and shall be further designated as "distilled" or "compounded," as the case may be.

(d) Gin contains at least 40 per cent ethyl alcohol by volume.

(e) The term flavoring of the dry gins are produced by juniper berries, coriander seed, fennel seed, sweet orange, and cassia bark.[2]

CORDIALS AND LIQUEURS

(a) Cordials and liqueurs are products obtained by mixing or redistilling neutral spirits, brandy, gin, or other distilled spirits with or over fruits, flowers, plants, or pure juices therefrom, or other natural flavoring materials, or with extracts derived from infusion, percolation, or maceration of such materials, and to which sugar or dextrose or both have been added in an amount not less than 2½% by weight of the finished product. Sweetening or imitation flavoring materials shall not be included.

(b) "Sloe gin" is a cordial or liqueur with the main characteristic flavoring derived from sloe berries.

(c) Cordials and liqueurs shall not be designated as "distilled" or "compounded."

(d) Dry cordials and dry liqueurs. The designation of a cordial or liqueur may include the word "dry" if the added sugar and dextrose are less than 10%, by weight of the finished product.[3]

---

[1] U. S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Sec. M, Sec. 30 (June 3, 1936).

[2] Wyatt, S. L., Modern Gin Production, The Wine Liquor Journ., p. 12, April, 1934.

[3] U. S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Sec. 31, Sec. 31 (June 3, 1936).

References on the Distilling Industries

Rum

Arroyo, R.: Studies on Rum, *Research Bull. No. 5, Univ. Puerto Rico, Agr. Expt. Sta.*, Rio Piedras, P. R., December, 1945.

Tobie, W. C.: Improved Procedures in Determination of Aldehydes in Distilled Alcoholic Liquors with Schiff's Reagent, *Food Research*, **3**: 499 (1938).

———: Personal communication, 1939.

U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

U.S. Dept. of the Treasury, Federal Alcohol Administration (Note to bottlers and importers of distilled spirits), Feb. 28, 1936.

Valaer, P.: Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

Whiskey

Adams, A. B.: The Distillation of Whiskey, *Jour. Ind. Eng. Chem.*, **2**: 34 (1910).

Anon: A Modern Distillery, *Chem. & Met. Eng.*, 126-II to 129-II, November, 1942.

Anon: "Plant Operations," The Calvert Distilling Co., Louisville, Ky., 1946.

Association of Official Agricultural Chemists: "Official and Tentative Methods of Analysis," 6th ed., Washington, D. C., 1945.

Boruff, C. S.: "Microbiology in a Modern Whiskey Distillery," p. 333, Abstracts of Communications, Third International Congress for Microbiology, September, 1939.

Brown, T. F.: Whiskey Distilling, *Am. Wine Liquor Jour.* (September, 1937).

——— and H. H. Schopmeyer: The Production of Bourbon, *Am. Wine Liquor Jour.*, p. 54 (December, 1935).

Foth, G.: "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.

Gallagher, M., P. Kolachov, and H. F. Willkie: Whiskey Losses during Aging, *Ind. Eng. Chem.*, **34**: 992–995 (1942).

Hirsch, I.: Manufacture of Whiskey, Brandy and Cordials, 2d ed., Sherman Engineering Co., Newark, N. J.

Kolachov, P.: The Distilling Industry in War and Peace, *Trans. Am. Assoc. Cereal Chemists*, **2** (No. 3): 14–23 (1944).

Owen, W. L.: The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine Liquor Jour.* (June, 1936).

Schicktanz, S. T., and A. D. Etienne: Acid Content of Whiskey. Variation with Age and Dilution, *Ind. Eng. Chem.*, **29**: 157–159 (1937).

Schmitt, R. O., P. J. Kolachov, and H. F. Willkie: Processing Equipment for Whiskey Filtration, *Ind. Eng. Chem.*, **34**: 1135–1138 (1942).

Unger, E. D.: Recent Advances in Alcohol Distillation Methods, *Chem. Inds.*, 778–780, May, 1946.

U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Relating to Labeling and Advertising of Distilled Spirits, as amended to Mar. 1, 1939, 1939.

U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Amendment 5, Feb. 28, 1938; Amendment 6, June 8, 1938.

U.S. Dept. of the Treasury, Federal Alcohol Administration, Notice to Bottlers and Importers, Feb. 28, 1936.

U.S. Dept. of the Treasury, Federal Alcohol Administration, A Digest of Interpretations of Regulations 5 Relating to Labeling and Advertising of Distilled Spirits, January, 1937.

VALAER, P., and W. H. FRAZIER: Changes in Whiskey Stored for Four Years, *Ind. Eng. Chem.*, **28**: 92 (1936).

WILLKIE, H. F.: Distillery Processes, *Chem. & Met. Eng.*, May, 1945.

WOODMAN, A. G.: "Food Analysis," 4th ed., McGraw-Hill Book Company, Inc., New York, 1941.

GENERAL

BARRON, H.: "Distillation of Alcohol," Joseph E. Seagram & Sons Co., Inc., Louisville, Ky., 1944.

BEAVENS, E. A., H. E. GORESLINE, and E. K. NELSON: Silver in the Artificial Aging of Brandies, *Ind. Eng. Chem.*, **29**: 623 (1937).

GORESLINE, H. E.: "List of Selected References on Distilled Liquors and Alcohol," U.S. Dept. Agr., Bur. Chem. and Soils, Food Research Division, July, 1936.

GUYMON, J. F., N. E. TOLBERT, and M. A. AMERINE: Studies with Brandy. I. pH, *Food Research*, **8** (No. 3): 224-230 (1943).

HERSTEIN, K. M., and T. C. GREGORY: "Wines and Liquors," D. Van Nostrand Company, Inc., New York, 1935.

JACOBSEN, E.: "Handbuch für die getranke-industrie, getranke-fabriken und den getranke-gross-betrieb," Paul Parey, Berlin, 1925.

JOSLYN, M. A., and M. A. AMERINE: Commercial Production of Brandies, *Bull.* 652, *Univ. Calif., Agr. Expt. Sta.*, Berkeley, September, 1941.

MARILLER, C.: "Distillation et rectification des liquides industriels," Dunod, Paris, 1925.

TOBIE, W. C.: $^1/_{10}$ of a degree for accurate proofing, *Am. Wine Liquor Jour.*, p. 17 (April, 1939).

TOLBERT, N. E., M. A. AMERINE, and J. F. GUYMON: Studies with Brandy. II. Tannin, *Food Research*, **8** (No. 3): 231-236 (1943).

Some Patents

FARRIER, L. H., and R. H. HARLIN: Apparatus for Purifying and Aging Liquor, U.S. Patent 2,108,661, Feb. 15, 1938.

KRAUSE, G. A.: Process for Artificially Aging Alcoholic Liquids and Perfumes, U.S. Patent 2,109,151, Feb. 22, 1938.

LIEBMANN, A. J.: Method of Making Gin, U.S. Patent 2,152,318, Mar. 28, 1939.

NAGLE, C. D.: Aging Device for the Contents of Barrels, Casks, or the Like, U.S. Patent 2,135,622, Nov. 8, 1938.

REIMAN, C. K.: Aging Distilled Alcoholic Spirits, U.S. Patent 2,132,435, Oct. 11, 1938

SHAPIRO, A.: Process of Making Liquors, U.S. Patent 2,160,036, May 30, 1939.

WILKEN, C. J.: Method of Producing Yeast Mash, U.S. Patent 2,132,091, Oct. 4, 1938

CHAPTER IX

PRODUCTION OF YEAST AND YEAST PRODUCTS

BAKERS' YEAST

Development of Industry.—The baking of bread has been practiced for centuries. Egyptian, Jewish, Greek, and Roman histories reveal many interesting facts concerning baking, brewing, and wine making. The Bible suggests a method for preparing leavened bread.

Mason, an Englishman, is said to have prepared the first compressed yeast in 1792.[1] No hops were used in its manufacture. Shortly thereafter, compressed yeast was known in Holland and in Germany, as well as in England. Indeed, there is some evidence that compressed yeast was made in Holland as early as 1781.

Leavened bread was prepared for many years by mixing a portion of leftover dough with the main batch and permitting the mix to stand for a while to undergo fermentation. A more modern practice was to mix residual yeast, from the brewery or distillery, with the dough. Such yeast often possessed a bitter taste due to the use of hops in malt-beverage manufacture.

The Vienna Process.—Some[2] consider that the compressed-yeast industry started with the development of the Vienna process, a modified brewing operation, about 1860. In this process, kiln-dried malt and corn were ground, mixed with water, and mashed (see chapter on Brewing). The unfiltered mash was pitched and permitted to ferment. Machines separated the portions of grain from the yeast by a process of sifting. Yields of 10 to 14 per cent on the basis of the grain were obtained along with a considerable amount (about 30 per cent) of ethyl alcohol.

Since compressed-yeast manufacturers are interested in the yield of yeast and not the alcohol, improvements in this method were made from time to time. A process for aerating mashes for yeast production was developed by Marquardt in 1879. Yields of yeast were materially increased, while those of alcohol were decreased. The filtration of the wort, the use of very dilute worts, carefully regulated aeration, and fairly low temperatures were later modifications that still further improved the process.

[1] FREY, C. N., *Ind. Eng. Chem.*, **22**: 1154 (1930).

[2] FREY, C. N., G. W. KIRBY, and A. SCHULTZ, *Ind. Eng. Chem.*, **28**: 879 (1936).

Many of the modern methods of compressed-yeast manufacture are protected by patents.

Soon after the commencement of World War I, research was carried out in Germany which led to the development of some of the modern methods of manufacture. The high cost and scarcity of grains had much to do with the instigation of this research.

Some General Considerations Concerning the Production of Yeast. *Yeast.*—Selected strains of *Saccharomyces cerevisiae* are used in the manufacture of compressed yeast. The baker depends upon the yeast to bring about definite changes in structure and flavor in the dough during a given amount of time. Failure to accomplish these changes may result in considerable loss of money. Therefore a vigorous strain that shows uniform and constant characteristics should be used for the manufacture of compressed yeast.

Nutrient Materials.—Carbon, necessary for growth and energy, is generally obtained from the sugars of the mashed grains or the molasses, although lactic acid, amino acids, and even ethyl alcohol may be used under certain conditions.

Nitrogen is supplied commonly by the use of malt or mashed cereal grains, or by ammonium salts. Peptones, peptides, amino acids, and ammonia are absorbed by the yeast and assimilated. Nitrates and nitrites, however, are not used by yeast.

Ammonium lactate, accordingly, may furnish both carbon and nitrogen.

Soybeans, freed of the bitter principles, sugar-beet juice, and various other substances have been proposed either to increase the yield of yeast or to act as the source of nutrient material.[1]

Concentration of Sugar.—The concentration of sugar is maintained at a low level in the fermentation (propagation) tank in order to favor its utilization for the manufacture of yeast cells rather than for the production of ethyl alcohol. The usual concentrations in the fermenter vary from about 0.5 to 1.5 per cent, depending on the process. The concentration of sugar in the wort is maintained at this low level throughout the period of production regardless of whether the latter is on a semicontinuous or continuous basis. This is accomplished by admitting a concentrated wort at a predetermined constant or logarithmically increasing slow rate.

Use of Lactic Acid Bacteria.—Frequently in the manufacture of yeast from cereal grain, the filtered mash may be inoculated with a culture of *Lactobacillus delbrueckii* and maintained for a while at a temperature of 50 to 52°C. to favor the production of lactic acid. Yeasts grow well in the

[1] CHRZASZCZ, T., and J. JANICKI, *Chem. and Ind.*, **55**: 884 (1936).

media containing lactic acid, while contamination, especially with butyric acid organisms, is reduced. The presence of lactic acid in the yeast cake inhibits the development of bacteria.

Aeration.—The proper aeration of the mash is of great importance in the manufacture of bakers' yeast. It is accomplished by passing air bubbles of suitable size through the entire medium.

The surface area of a given volume of bubbles of air in a liquid increases as the sizes of the bubbles become smaller. There is thus a larger contacting surface with small-sized bubbles than with large ones and a longer period of contact with the medium or wort. A smaller quantity of air is required when the bubbles used are of small size, but it costs more to produce very fine bubbles. Thus, the yeast manufacturer must select a desirable course, in which efficiency of aeration is high but not too costly. The bubbles used commercially vary from 0.0001 to 1 in. in diameter, according to de Becze and Liebmann,[1] who have thoroughly discussed the subject of aeration.

Air is introduced near the bottom of the fermentation tank and the aeration network should be of such nature that there is uniform distribution of air over the entire cross section of the tank.

The air requirements for a wide and shallow fermentor tank will be much different from those of a narrow, tall tank, for the longer a bubble of air is in contact with the medium, the more effective it is. Based on the yeast produced, a considerably larger amount of air is required for a small fermentor than for a large one.

de Becze and Liebmann reported that the correct evaluation of air requirements must be based on information available concerning the height of the wort at any time (obtained by reference to a chart or graph); the concentration of yeast at any time (by graph); the yield of yeast, per unit of raw material (by graph); and the ratio of the amount of new yeast to seed yeast.

Air requirements are generally reported in terms of cubic feet per (1) gallon of wort, (2) square foot of wort or fermentor bottom surface, (3) pound of yeast in 100 gal. of wort, (4) pound of yeast produced, (5) ton of total molasses, or (6) bushel of grain mashed; or milliliters, liters, or cubic meters per unit volume of medium, etc.

Aerators are of many types. For example, they may be plates, tubes, candles, spargers, or false bottoms. They may be of the fixed or movable type. They may be constructed of noncorrosive metal, ceramic substances, hard or soft rubber, porous carbon, sintered glass, canvas, or other substances. For a review of this subject the reader is referred to the article by de Becze and Liebmann.

[1] DE BECZE, G. and A. J. LIEBMANN, *Ind. Eng. Chem.*, **36**: 882 (1944).

Function of Oxygen.—The exact function of oxygen in the growth of yeast is not known. Very likely its action may be due to several factors: inhibition of fermentation and increase of respiration, agitation of the medium, removal of toxic end products, and stimulation of vegetative growth.

pH.—The pH is generally adjusted to 4.4 to 4.6. However, slightly lower or slightly higher values may be used under certain conditions.

The pH is maintained by one or more methods. One way is to check the pH every 30 min. or so and adjust by the addition of ammonia or sulphuric acid as indicated. Another way is to control automatically. Much may be done to control pH simply by using a suitable proportion of nutrients in the strong wort.

Temperature.—The temperature is generally started at 25 or 26°C. It is usually held fairly close to this level during the early part of the fermentation and gradually permitted to rise to 30°C. towards the end of the fermentation. Cooling and heating coils are an essential part of the fermentation-tank equipment.

Preserving the Yeast.—The use of a low temperature is necessary for the storage of compressed yeast. Molds and bacteria cause the cake to deteriorate rapidly at room temperature and autolysis is likely to take place.

Various methods for improving the keeping qualities of yeasts have been proposed. For example, it has been suggested[1] that washing the yeast for 1 hr. with a solution of ethyl, propyl, isopropyl, butyl, or amyl alcohol before pressing improves the keeping quality. The use of colloids - pectin, agar, gelatin, gum tragacanth, dextrin, algae extract—to remove the water from yeast, followed by drying to a definite moisture content at a temperature that will not injure the yeast, has also been proposed.

Molasses-Ammonia Process.—In the molasses-ammonia process, molasses (cane and sugar beet) is used as the source of carbon and mineral elements for the growth of the yeast, while the nitrogen is supplied by ammonia, or ammonium sulphate.

The molasses mash is carefully prepared. If the molasses is deficient in phosphorus, ammonium or calcium phosphate is added. Phophorus is important in the synthesis of nucleoproteins by the yeast and in buffering the medium. It stimulates growth also.

In order to produce a clear solution, the molasses wort is filtered.

The pH of the clear, highly diluted mash is adjusted. The wort is then inoculated with a starter of yeast, which has been carefully prepared from a single cell of the selected yeast.

[1] British Patent 405,395, 1934.

During operation, several factors must be carefully controlled. The most important of these are the aeration, the pH, the concentration of the available sugar, and the temperature.

The availability of a large supply of air stimulates the production of yeast cells. All the air used must be sterilized before passage through the mash.

The pH is maintained within a definite range, that most favorable for yeast growth (about 4.4), during the entire growth period by the addition of sulphuric acid or ammonium hydroxide. As the ammonia is used up, the mash tends to become more acid in reaction. The addition of ammonium hydroxide not only neutralizes some of the acid but also supplies more ammonium salts for the nitrogenous needs of the growing yeast.

During the process, measured portions of the molasses wort are added at specified time intervals in such quantities that the yeast produces but very little alcohol from the sugar.

It is essential to control the temperature of the mash during the growth of the yeast. This may be done by the use of cooling coils set on the inside of the covered vats, which usually have capacities for several thousand gallons. The optimum temperature for the growth of the yeast strain being used is maintained.

At the end of the growing process, yeast is separated from the wort by the use of centrifugals (or by filtration). The yeast thus obtained is washed repeatedly, until the wash water appears to be quite clear. It is then pressed.

A small amount of flour (potato, tapioca, or cereal) may or may not be added to the yeast. The flour absorbs some of the moisture from the yeast, increases the friability, and aids in molding the yeast into small cakes. Much yeast for the baking industries is packaged without the incorporation of starch.

A Procedure Employed in Germany.—Bakers' yeast was produced in one German plant by the following procedure[1]: Beet molasses is diluted to 30°Bé and adjusted to pH 4.5. It is then sterilized by heating at 105°C. for 1 hr. The solution is allowed to stand for 4 to 6 hr. during which time suspended solids settle out and the temperature drops to 90°C. It is filtered. Nutrients, in the form of 110 kg. of ammonium sulphate and 250 kg. of superphosphate for each 7,000 kg. of yeast produced, are added. This mixture is held in storage tanks and later fed at a slow predetermined rate to the fermentation tank.

The inoculum is prepared as follows: A strain of *S. cerevisiae*, especially suitable for the type of molasses being used, is grown on a slant. This

[1] BALLS, A. K., *Fiat Final Report No.* 277, Oct. 3, 1945.

culture is used to inoculate a 1-liter mash. The size of the inoculum is progressively increased by successive transfer through 50-, 200-, 1,000-, and 15,000-liter mashes. Finally a 90,000-liter inoculum is prepared which is used to inoculate six large fermentors of the type described below.

Fermentation (propagation) tanks are constructed of steel and are of 200,000-liter capacity. To start a fermentation, 20,000 liters of water are run into the tank. Sterilized, nutrient molasses is added to the water until the sugar content reaches 1.5 per cent. The inoculum is then added and aeration commenced. The feed of the sterile 30°Bé molasses is then adjusted so that the sugar concentration is kept at 1.5 per cent. The temperature is maintained at 25 to 30°C. The fermentation is over in 10 to 11 hr.

The aerating system consists of perforated copper tubes located at the bottom of the fermentor. For each kilogram of yeast produced, 16 cu.m. of air are supplied. A supply of 4,000 cu.m. of air per hr. is necessary for 7,000 kg. of molasses. Crude lanolin is used as an antifoam agent.

The yeast is separated from the spent wort by means of Westphalia centrifuges. The cream so obtained is washed twice, cooled to 8 to 9°C., and pressed in a plate-and-frame press. The resultant cake contains 31 per cent solids. It is diluted with water to 25 per cent solids before being packaged.

Yeast from Sulphite Liquor by the Heijkenskjöld Method.[1]—Sulphite liquor, mixed with a small quantity of molasses, serves as the source of raw material for the manufacture of yeast by the Heijkenskjöld method.

The first step in this process consists of adjusting the hot sulphite liquor to a pH of 6.0 to 6.5 with lime, powdered limestone, and sodium carbonate, while the liquor is being aerated in tanks. After the neutralization, the liquor is permitted to stand, in order that the precipitates may settle out. The clear supernatant liquor is decanted from the tanks and cooled to 28 to 30°C. by passage through coolers and is then ready for use in the manufacture of yeast.

The initial mash contains molasses mixed with sulphite liquor to yield a sugar concentration of 3 to 5 per cent. Malt sprouts may be added to this mash, if desired, to supply organic nitrogen and other nutrient substances. The mash is now inoculated with the seed yeast, a strain of *Saccharomyces cerevisiae* ("Rasse 12" of the Gärungsinstitut of Berlin is used in Finland and at the Best plant in Liverpool, Nova Scotia[2]).

Sulphite liquor is fed into the mash after the yeast has commenced to develop new cells. Ammonium sulphate and phosphates are added during the course of the process to furnish nutrient material and to main-

[1] Eweson, E. W., *Chem. Industries*, **38**: 573 (1936).
[2] *Ibid.*

tain the desired reaction. Large quantities of filtered air are supplied, the air removing the carbon dioxide and other gases formed besides carrying out other functions. After the propagation is complete, the yeast is washed repeatedly. The final product is light in color, of high purity, and neutral in taste.[1]

The sulphite liquor used in this process contains approximately 25 kg. of sugar per 1,000 kg. of liquor, and of this amount about 16 kg. are fermentable. The sugar concentration is thus very low and would not favor the production of alcohol in large amounts.

An average yield of 160 per cent is obtained on the basis of the fermentable sugar, yields varying from 40 to 55 lb. for each ton of waste sulphite liquor.

TABLE 51.—COMPOSITIONS OF SOME MEDIA FOR GROWTH OF YEAST[1]

Grain-wort Medium		
Corn	2	kg.
Malt	1.5	kg.
Sprouts	1.5	kg.
Concentrated hydrochloric acid	10	cc.
Tap water, to make	30	liters
Molasses-salts Medium		
Beet molasses (crude)	150	g.
Calcium phosphate	1	g.
Ammonium monohydrogen phosphate	1	g.
Urea	2	g.
Concentrated sulphuric acid	2	cc.
Tap water, to make	1	liter
Glucose-salts Medium		
Commercial glucose	70	g.
Sodium chloride	2	g.
Magnesium sulphate	2	g.
Calcium chloride	0.4	g.
Potassium pyrophosphate	2	g.
Ferric pyrophosphate	0.1	g.
Ammonium chloride	0.15	g.
Copper sulphate	Trace	
Urea (commercial)	2.4	g.
Tap water, to make	1	liter

[1] PAUCEK, P. L., W. H. PETERSON, and C. A. ELVEHJEM, *Ind. Eng. Chem.*, **29**: 536 (1937).

Some Media for Yeast Propagation and Their Preparation.—Table 51 presents the compositions of some media for the growth of yeast. They are prepared as follows:

1. *Grain-wort Medium.*—The finely ground corn is autoclaved at a pressure of 15 lb. per sq. in. for 0.5 hr. in the presence of about 8 liters of water. At the same time the malt and sprouts are mixed in 20 liters of water, 5 cc. of concentrated HCl is added, and the mixture is allowed

[1] *Ibid.*

to soak at 25°C. for 0.5 hr. Approximately one-half of the cooked corn is added to this malt-sprouts mixture. The temperature is then raised to 50°C. and maintained at this level for 0.5 hr. Next the rest of the corn plus an additional 5 cc. of concentrated HCl are added, and the temperature is raised to 62°C. and kept constant until the iodine test for starch is negative. The mash is filtered through burlap bags, and the residue is washed once with warm water. The combined filtrate is made up to 30 liters with water and autoclaved at a pressure of 15 lb. per sq. in. for 45 min. The resultant wort should contain 4.5 to 5 per cent of reducing sugar (as glucose) and have a pH of 4.4.

2. *Molasses-salts Medium.*—The molasses, calcium phosphate, and sulphuric acid are sterilized together after dissolving them in water. Sterilized urea and ammonium monohydrogen phosphate are added to the cooled solution, and the pH is adjusted to 4.4.

3. *Glucose-salts Medium.*—Solutions of glucose, sodium chloride, magnesium sulphate, calcium chloride, and copper sulphate are autoclaved at a pressure of 15 lb. per sq. in. for 1 hr. at a pH of 3.5. A sterilized solution of potassium and ferric pyrophosphates is added to the cooled main mash. If a precipitate forms it is dissolved by adding some sterilized 40 per cent sulphuric acid. A sterilized solution of ammonium chloride and urea is added finally to the main mash, and the pH is adjusted to 4.4 with sterilized 40 per cent sodium hydroxide (NaOH). Although the ratio of carbon to nitrogen in the preceding medium is 25:1, the ratio can be reduced by adding increasing quantities of ammonium chloride and urea.

Yields.—Pavcek, Peterson, and Elvehjem have reported the following yields of dry yeast grown on the media indicated:

TABLE 52.—YIELDS OF DRY YEAST[1] FROM VARIOUS MEDIA[2]

Type of yeast	Grain medium, per cent	Molasses-salts medium, per cent	Glucose-salts medium, per cent
Bakers' Yeast *A*	24.3	34.6	18.0
Bakers' Yeast *B*	42.5	33.6	34.3
Brewers' Yeast *A*	34.6	42.7	29.0
Brewers' Yeast *A* (autoclaved medium)	32.2		
Saccharomyces logos	33.1	28.0	21.4
Willia anomala	21.4	28.6	11.4
Endomyces vernalis	40.9	33.6	30.5

[1] Dry yeast is based on glucose fermented.

[2] PAVCEK, P. L., W. H. PETERSON, and C. A. ELVEHJEM, *Ind. Eng. Chem.*, **30**: 802 (1938).

without aeration the yields were of the magnitude of only one-tenth of those with aeration.

Active Dry Yeast.—This is a dried bakers' yeast. It is manufactured by the usual processes and dried to a moisture content of about 8 per cent. Such yeast may be stored for several months without appreciable loss of its stability. Large quantities were produced for use by the Armed forces

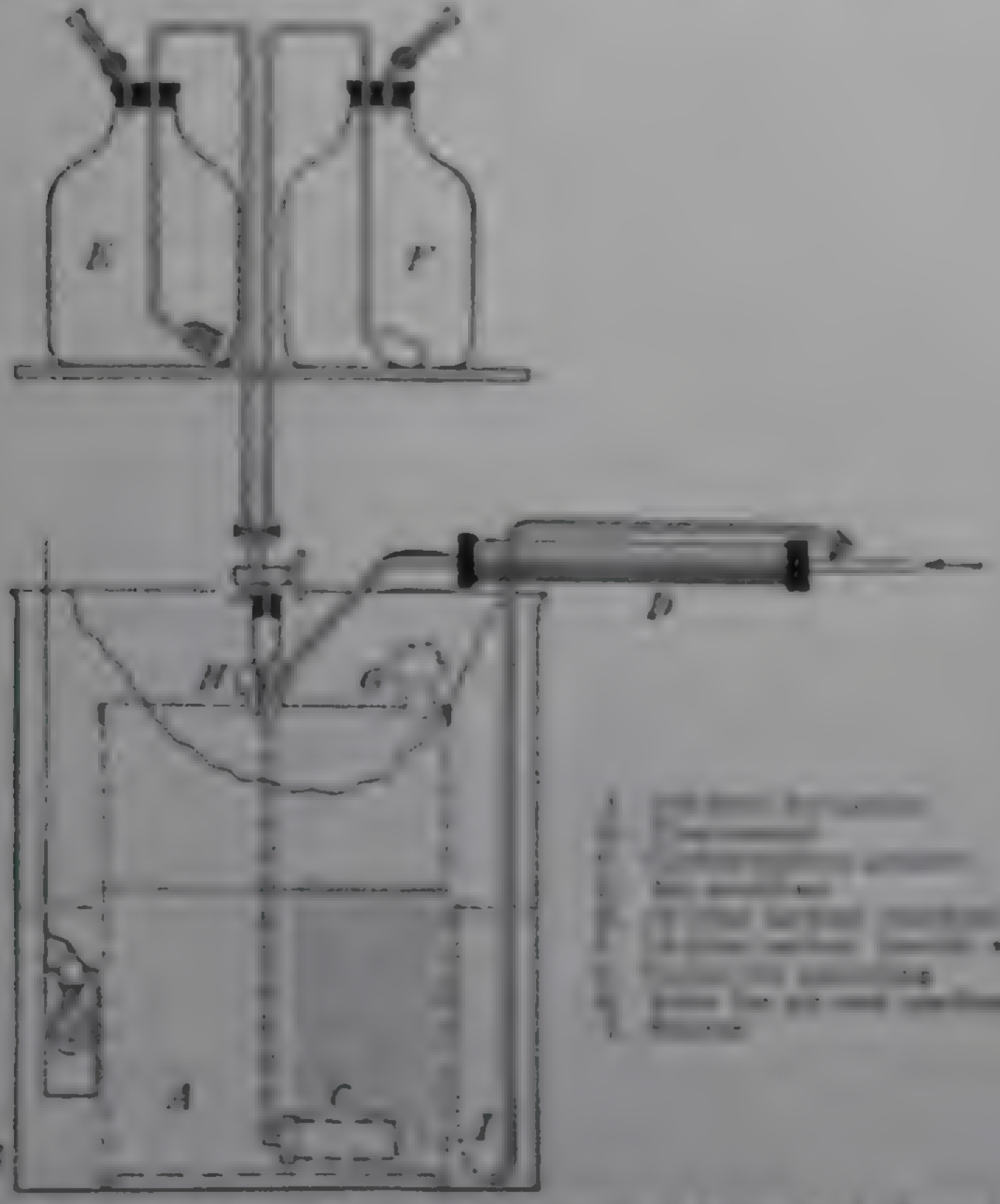


Fig. 37.—Apparatus for growing yeast. [Courtesy of Prof. P. L. Brunel, W. G. Rogers and C. A. Elvehjem, Ind. Eng. Chem. 29: 1937]

and other agencies during World War II. [illegible] lb. in 1943, [illegible] lb. in 1944, and [illegible] lb. in 1945.

Florida Yeast.—This is a dried yeast and is produced from fresh press-yeast, ordinarily produced with higher aeration than the usual yeast to encourage glycogen formation.

The press-yeast is put through a folding machine and spread 1 to 1.5 cm. deep in trays. It is dried by passing air at 30° to 35°C. over the trays for 36 to 48 hr. (or 28°C. for 48 to 50 hr.). The resulting product is the

[illegible]
[illegible]

product is 10 per cent. Four grams of the product has the same value as 5 g. of fresh yeast. The product lasts for 8 to 12 months.

Apparatus for Growing Yeast.—Figure 37 shows an apparatus suitable for growing yeast.

FOOD AND FODDER YEASTS

Food yeast is propagated primarily for human consumption as a food constituent. Fodder yeast is produced for animal feeding. Both are produced under similar conditions, except for the raw materials used and the final treatment of the products.

Food yeast is a rich source of protein and vitamins of the B-complex. In the dry state, it usually contains about 50 per cent of protein and when used in the proper proportions with other foods it makes a satisfactory and nutritious supplement to the diet of persons living under conditions where there may be a shortage of animal proteins or vitamins of the B-complex.

The manufacture of fodder yeast is of interest to those desiring to convert waste, surplus, or low-cost carbohydrate materials into products of value for feeding cattle, hogs, or other farm animals. The utilization of waste sulphite liquors for fodder yeast production, for example, results in the extension of animal foods and at the same time aids in the alleviation of stream pollution.

Food yeast was manufactured and used to some extent by the Germans during World War I. Several thousand tons were produced and consumed in Germany during World War II,[1] the raw materials being sulphite liquor or wood hydrolyzates. The Colonial Office of Great Britain undertook the construction of a large plant in Jamaica for producing food yeast early during the latter war.[2] India is much interested in this subject, as are other countries.[3]

General Details of Production.—The following paragraphs contain information of a general nature concerning the production of food and fodder yeasts.

Organisms Used.—The organisms most commonly employed for food or fodder yeast production is a strain of *Torulopsis utilis*. However, other yeasts have been used commercially in Germany, such as *Torula pulcherima, Monilia candida, Candida arborea,* mixed *Torula,* and *Oidium lactis.*[4]

[1] SAEMAN, J. F., E. G. LOCKE, and G. K. DICKERMAN, *Fiat Final Report* No. 499, Nov. 14, 1945.

[2] Colonial Food Yeast Ltd., Food Yeast, May, 1944.

[3] THAYSEN, A. C., *Food*, p. 116, May, 1945.

[4] SAEMAN, LOCKE, and DICKERMAN, *loc. cit.*

A number of other yeasts have been studied, including *Mycotorula lipolytica*, *Hansenula anomala*, *H. suaveolens*, *Candida tropicalis*, *Torulopsis candida*, and *Torula lactosa*.

Torulopsis utilis is particularly desirable on account of its high protein content, its vitamins of the B-complex, and its ability to utilize hexoses, pentoses, acetic acid, and certain other constituents which may be found in the acid hydrolyzates from wood or other cellulosic materials. It normally produces but little alcohol.

There are a number of strains of *T. utilis*. One, known as *T. utilis major*, produces larger cells than the usual strains; while another strain, *T. utilis thermophila* grows well at higher temperatures than most food yeasts.

The biochemistry of *T. utilis* has been studied by Fink and Just. Their findings have been published in a series of articles appearing in *Biochemische Zeitschrift*.[1]

Studies concerning the metabolism of growing *T. utilis* under aerobic conditions have been carried out by Sperber.[2]

REQUIREMENTS OF THE YEAST.—The yeast used for food production should possess the following characteristics: It should be one of high protein and vitamin content. It should grow rapidly, produce high yields, and be palatable. It should be able to grow under the conditions and on the substrates that are available.

ACCLIMATIZATION.—As a rule a faster rate of growth and larger yields of food yeast are obtained from hydrolyzed wood and certain other substrates, if the yeast has become acclimatized to the raw material by being repeatedly cultured in it.

SIZE OF INOCULUM.—Usually the concentration of yeast cells in the inoculum is of such magnitude as to give 100,000,000 to 200,000,000 cells in the propagation (fermentation) medium.

Raw Materials.—The raw material used will depend upon a number of factors, the most important of which are availability, cost, and assimilability. In Germany, waste sulphite liquor, particularly from beechwood, was used in six or more plants for food yeast production during World War II. In addition, five plants produced yeast from the acid-hydrolyzed wood obtained by the Bergius and Scholler methods. Molasses and whey are normally available as low-priced carbohydrate-containing materials. Hydrolyzed grains, citrus fruit wastes, wood sugar stillage, and carob beans are other sources. Actually a large variety of raw materials may be used after any necessary pretreatments.

[1] FINK, H., und F. JUST, *Biochem. Zeit.*, **300**: 84 (1938); **303**: 1,234,404 (1939); **312**: 390 (1942); etc.

[2] SPERBER, E., *Arkiv Kemi, Mineral Geol.*, **21** (N:03): 1–136 (1945).

Lechner studied the utilization of pentoses[1] and other compounds by *T. utilis*. He found that xylose was readily assimilated,[2] but that rhamnose and glucuronic acid were not utilized.[3]

SUGAR CONCENTRATION.—The initial sugar concentration in the propagation tank or container is usually adjusted to 1 per cent or less.[4,5,6] In continuous processes, the concentration of the feed may be much higher.

NITROGEN SOURCES.—Nitrogen may be applied in a number of forms. In Germany, ammonium sulphate and ammonia were used extensively in commercial operations. It was also supplied as diammonium phosphate.[7] Urea has been found to be a suitable source of nitrogen by investigators in this country.[5,6]

PHOSPHORUS SOURCES.—Diammonium phosphate and superphosphate were used commercially in Germany.[8] Potassium dihydrogen phosphate or superphosphate in equivalent amounts has been used successfully in the United States.[9]

OTHER NUTRIENTS.—In Germany, potassium chloride and magnesium sulphate were used in the production of yeasts from wood sugar. Magnesium chloride has also been used as a source of magnesium on an industrial basis.

pH.—The initial pH is generally adjusted to approximately 4.5, depending upon the raw materials used and the conditions of fermentation. Vincenty and Lewis, Stubbs, and Noble adjusted their media to an initial pH of 4 to 5; Peterson, Snell, and Frazier (1945), to an initial pH of 4.5 to 5.5; and Agarwal, Singh, King, and Peterson (1947), to an initial pH of 4.6.

Temperature.—The temperature normally used for food and fodder yeast production is 30°C. Where it is difficult to maintain the temperature at this optimum, special strains of yeasts that grow well at higher temperatures (35 ± °C.) may be used.

Aeration.—Provided that other conditions are satisfactory, aeration is probably the most important factor in the successful production of

[1] LECHNER, R., *Angew. Chem.*, **53**: 163 (1940).
[2] LECHNER, R., *Biochem. Zeit.*, **304**: 84 (1940).
[3] LECHNER, R., *Biochem. Zeit.*, **306**: 218 (1940).
[4] VINCENTY, C., Unpublished research report, M.I.T., Feb. 18, 1944.
[5] PETERSON, W. H., J. F. SNELL, and W. C. FRAZIER, *Ind. Eng. Chem.*, **37**: 30 (1945).
[6] AGARWAL, P. N., K. SINGH, P. S. KING, and W. H. PETERSON, *Arch. Biochem.*, **14** (Nos. 1 and 2): 105 (1947).
[7] SAEMAN, LOCKE, and DICKERMAN, *loc. cit.*
[8] *Ibid.*
[9] AGARWAL, SINGH, KING, and PETERSON, *loc. cit.*

TABLE 53.—YIELDS AND PROTEIN CONTENTS OF YEASTS GROWN IN DIFFERENT MEDIA

Yeast	Medium	Dry yeast yield, per cent	Protein content, per cent	Reference
Torulopsis utilis No. 3	Wood hydrolyzates	35–40*		PETERSON, SNELL & FRAZIER, *Ind. Eng. Chem.*, **37**: 30 (1945)
Torulopsis utilis No. 3	Wood hydrolyzates	30.5–52.6†	51.9–58.6	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind. Eng. Chem.*, **40**: 1220 (1948).
Torulopsis utilis No. 3	Sulphite liquor	29.6–39.2†	51.0–52.7	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind. Eng. Chem.*, **40**: 1220 (1948).
Torulopsis utilis No. 3	Fermentation residue	47.0–52.0†	50.0	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind. Eng. Chem.*, **40**: 1220 (1948).
Torulopsis utilis No. 3	Wood sugar stillage	53–63‡	52.9	KURTH & CHELDELIN, *Ind. Eng. Chem.*, **38**: 617 (1946).
T. utilis (Anheuser-Busch)	Molasses (cane) Puerto Rican	45.1–48.4§	33.1–50.8	VINCENTY, M. I. T. Research Report, Feb. 18, 1944.
T. utilis	Molasses (beet)	53.5–65.3†	43.7–60.6	AGARWAL, SINGH, KING & PETERSON, *Arch. Biochem.*, **14** (Nos. 1 & 2): 105 (1947).
T. utilis	Molasses	56†	Av. over 50**	LEWIS, STUBBS & NOBLE, *Arch. Biochem.*, **4** (No. 3): 389 (1944).
T. utilis	Fruit juice	53†	Av. over 50**	LEWIS, STUBBS & NOBLE, *Arch. Biochem.*, **4** (No. 3): 389 (1944).
Torulopsis utilis var. major	.....	59–62‡	56	THAYSEN & MORRIS, *Nature* **152** (No. 3852): 526 (1942).
Candida arborea	Molasses (beet and cane)	55–64†	38.8–49.4	AGARWAL, SINGH, KING & PETERSON, *Arch. Biochem.*, **14** (Nos. 1 & 2): 105 (1947).
Hansenula anomala	Wood hydrolyzates	35–40*	.....	PETERSON, SNELL, & FRAZIER, *Ind. Eng. Chem.*, **37**: 30 (1945).
H. suaveolens y-838	Wood sugar stillage	53–63‡	53.4	KURTH & CHELDELIN, *Ind. Eng. Chem.*, **38**: 617 (1946).
Mycotorula lipolytica P-13	Wood hydrolyzates	35–40*	.....	PETERSON, SNELL & FRAZIER, *Ind. Eng. Chem.*, **37**: 30 (1945).
Mycotorula lipolytica P-13	Wood sugar stillage	53–63‡	51.0	KURTH & CHELDELIN, *Ind. Eng. Chem.*, **38**: 617 (1946).
Oidium lactis	Molasses (beet and cane)	55.8–60.0†	31.2–41.9	AGARWAL, SINGH, KING & PETERSON, *Arch. Biochem.*, **14** (Nos. 1 & 2): 105 (1947).
Saccharomyces cerevisiae	Molasses (beet and cane)	42.7–54.3†	42.5–53.1	AGARWAL, SINGH, KING & PETERSON, *Arch. Biochem.*, **14** (Nos. 1 & 2): 105 (1947).

* Based on total reducing sugars.
† Based on total sugar.
‡ Based on sugar consumed.
§ Based on sugar in molasses.
** In most cases.

yeast. It is essential to supply an adequate volume of air in sufficiently fine bubbles to accomplish efficient oxygenation of the medium.

Foaming is ordinarily a problem in the production of yeast. It may be controlled by the use of special aeration and agitation devices, such as are shown in Figs. 40 and 43, or by the use of suitable antifoam agents. Peterson, Snell, and Frazier used Vegifat Y as a defoaming agent.

Yields.—The yields obtained depend upon the yeast used, the medium, and the conditions of propagation. Yields (dry yeast) of 53 to 64 per cent based on the sugar present are not uncommon. Table 53 summarizes some data concerning yields and the protein content of yeasts produced by different investigators.

Production from Various Raw Materials.—A few examples of production methods follow:

Production from Sulphite Liquors.—Food yeast was produced commercially from sulphite liquors in Germany during World War II, principally by the Waldhof process, which has been described in some detail by Saeman, Locke, and Dickerman.[1] The standard German yeast plant, using the Waldhof process, had a capacity for 1,000 tons of yeast (7 per cent moisture) per year. The process is a rapid, continuous one which employs mechanical aeration and foam-breaking equipment.[1]

The raw material is beech liquor, although spruce liquor is apparently just as suitable. It is neutralized with lime while undergoing aeration, clarified by a settling procedure, and cooled by means of a heat exchanger. Nutrients, such as diammonium phosphate, ammonia, potassium chloride, and magnesium sulphate, are added to the clarified liquor.

The medium is aerated by a mechanical device,[2] which consists of a wheel approximately 1.5 to 1.7 meters in diameter with air ducts leading from a central pipe to the periphery. The wheel is rotated at about 400 r.p.m. From 8 to 12 cu.m. of air are supplied for each kilogram of yeast manufactured.

The propagation of yeast is carried out at a temperature of 32°C. Since the process is an exothermic one, the fermentor (propagation tank) is supplied with cooling coils (refer to Fig. 38) and the feed solution is cooled to 20°C. in order to aid in the maintenance of the 32°C. temperature.

The nutrient solution is fed into the fermentor (usually of 300-cu.m. capacity) at a measured rate during the process, depending upon its sugar concentration and the capacity of the fermentor. The propagation time may be as short as 4.5 hr. when a 2.6 per cent sugar solution is fed into the fermentor at a rate of 12 cu.m. per hr.

[1] SAEMAN, LOCKE, and DICKERMAN, *op. cit.*
[2] *Ibid.*

The yeast suspension is removed continuously. Centrifuges concentrate the liquor to a maximum of 12 per cent. The yeast suspension is concentrated to a 20 per cent cake by a rotary filter. The yeast is then autolyzed and dried on a drum drier; or it may be autolyzed and concentrated (by evaporation) to 30 per cent dry substance and spray dried.

Harris and associates[1] and others have studied food yeast production from sulphite liquors.

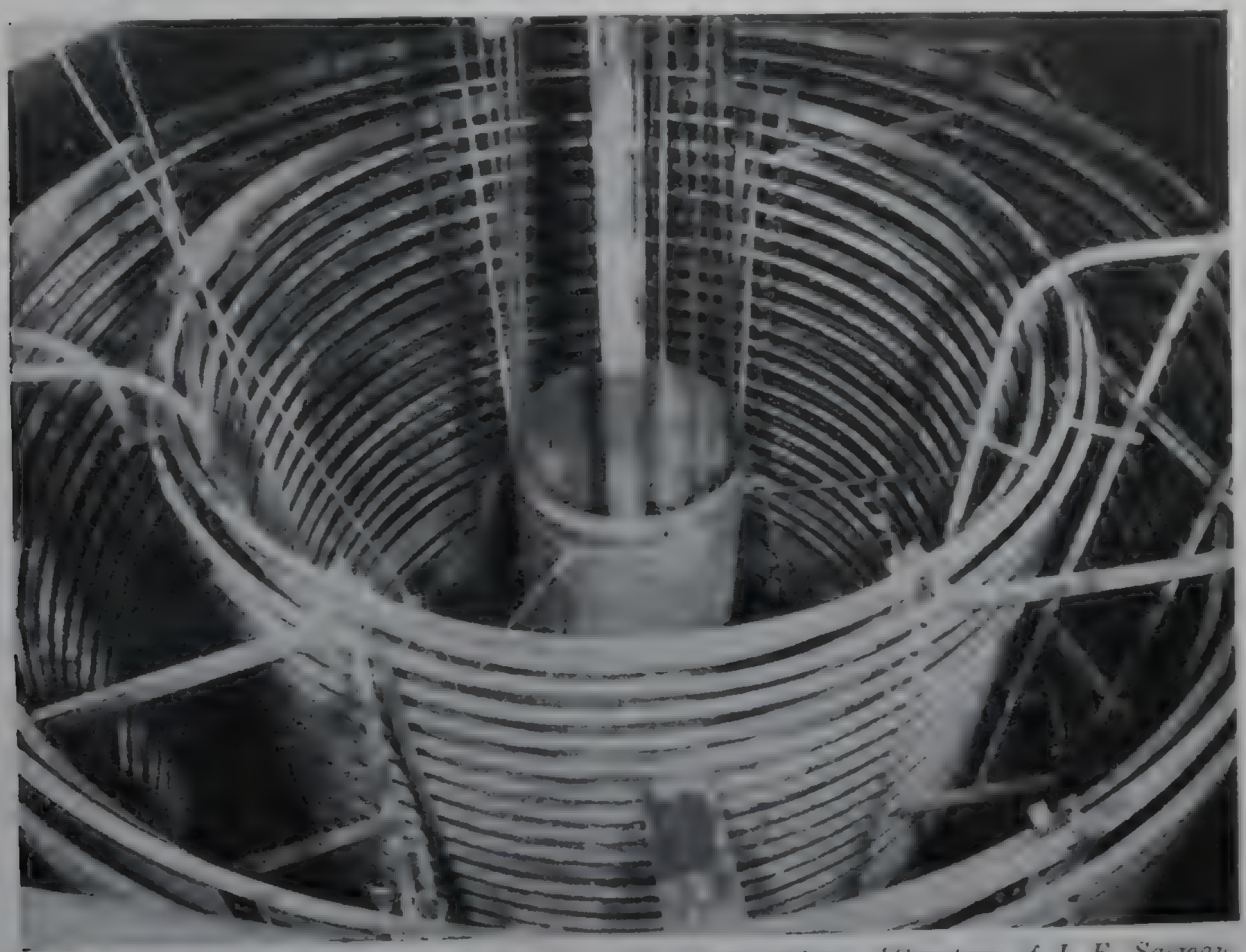


FIG. 38.—Cooling coils in fermentor for yeast propagation. [*Courtesy of J. F. Saeman, E. G. Locke, and G. K. Dickerman, Fiat Final Report* No. 499, Nov. 14, 1945; also *E. G. Locke, Pulp & Paper Ind.*, **20** (No. 1): 20 (1946).]

Fodder Yeast from Sulphite Liquor.—The production of fodder yeast from sulphite liquor on a laboratory scale has been described by Walker and Morgen.[2] The sulphite liquor was neutralized to pH 10.5 with lime, permitted to settle, filtered with the help of filter aid for sludge removal, neutralized to pH 3.5 with sulphuric acid, and filtered. The sugar concentration of different samples of liquor varied from 2 to 2.5 per cent.

[1] HARRIS, E. E., J. F. SAEMAN, R. R. MARQUARDT, M. L. HANNAN, and S. C. ROGERS, *Ind. Eng. Chem.*, **40** (No. 7): 1220 (1948).

[2] WALKER, R. D., JR. and R. A. MORGEN, *Paper Trade Jour.*, **123** (No. 6): 43 (1946).

Walker and Morgen found that optimum conditions were obtained for fodder yeast production with *Torulopsis utilis* var. *thermophilia* when the sulphite liquor contained 2.0 g. diammonium hydrogen phosphate per liter; the pH was 5.75 (5.5 to 6.0); the temperature was 30 to 35°C.; and 150 ml of air per minute per liter of sulphite liquor (based on the conditions and fermenter used) were employed. Turkey red oil, in a concentration of 0.1 to 0.2 per cent, was effective as an antifoam agent and apparently nontoxic.

During a 15-hr. period, 90 per cent of the reducing sugars were utilized and yeast concentrations of 500 to 700 million cells per milliliter were obtained (as high as 5 per cent by volume).

Inoculums (about 1 per cent) from wort agar slants or from sulphite liquor that was in state of active fermentation were used.

Harris, Hannan, and Marquardt[1] described the production of fodder yeast from sulphite liquor. They found that sulphur dioxide and other volatile substances could be removed by steam-stripping or by precipitation with lime. Steam-stripping is the more satisfactory method; however, the equipment used for this must be acid-resistant and is costly. After removal of the sulphur dioxide, the liquor is neutralized with lime to a pH of 4 to 4.5. The resultant precipitate of calcium sulphate, and any pulp fibers, are removed by filtration. In an alternate method, as is used in Germany, the sulphite liquor is first neutralized directly to a pH of 4.1 to precipitate both the sulphites and the sulphates, aerated, and then filtered. The necessary nitrogen and phosphate nutrients are added to the liquor, which is then inoculated with *T. utilis* No. 3. Propagation is carried out in the special fermentor described on page 267. Data and results of typical runs are shown in Table 54.

Production from Wood Sugar.—According to Saeman, Locke, and Dickerman,[2] 8,985 tons of yeast were produced in Germany in 1944 by five plants which used sugar obtained by the acid hydrolysis of wood. The wood sugar was produced by the Bergius and Scholler methods.

The process, as carried out at the Regensburg Vogelbusch plant, was as follows: Propagation was conducted in six 250-cu.m. Vogelbusch fermentors, each of which was equipped with internal cooling coils and a 20 KVA motor which drove the propeller at 8 r.p.m.

The organism used was *Torulopsis utilis*. The inoculum for a fermenter was obtained from a previous fermentation. On the basis of the sugar being used, a quantity equivalent to 10 to 20 per cent by weight of dry yeast was employed.

[1] Harris, E. E., M. L. Hannan, and R. R. Marquardt, *Paper Trade Jour.*, Nov. 27, 1947.

[2] Saeman, Locke and Dickerman, *loc. cit.*

TABLE 54.—CONTINUOUS PROPAGATION OF *Torula utilis*[1]

Series no.	Rate of feed per hour, liters	Air per pound of dry yeast, cubic feet	Reducing material used, per cent	Yield of dry yeast on sugar used, per cent	Protein content of yeast, per cent
Wood Hydrolyzate (Sugar content, 4.6 per cent)					
10	2.0	630	92.0	39.0	51.3
14	3.0	295	93.8	49.7	57.5
15	4.0	210	93.4	52.6	56.2
16	4.5	212	93.2	46.5	55.4
17	5.0	203	91.1	43.4	51.5
Sulphite Waste Liquor from Blow Pit Neutralized with Lime (Sugar content, 1.66 per cent)					
21	2.0	1,040	79.0	49.5	51.6
22	3.0	695	78.0	49.0	51.5
23	4.0	540	75.0	50.0	51.0
24	5.0	550	59.0	50.2	50.0
Sulphite Waste Liquor, Steam-stripped, Neutralized with Lime (Sugar content, 2.56 per cent)					
748	2	890	77.0	49.5	42.6
752	2	850	74.3	52.0	43.2
765	3	850	75.0	46.1	43.6
770	3	850	73.9	47.4	51.4
Evaporated Sulphite Waste Liquor, Neutralized with Lime (Sugar content, 12 per cent)					
783	3	.....	68.8	15.4	
784	3	.....	51.8	8.2	
Evaporated Sulphite Waste Liquor Diluted 1:1 with Water (Sugar content, 6.0 per cent)					
789	2	720	75.0	36.0	43.6
792	2	640	77.6	38.1	
778	3	505	75.9	32.6	
781	3	500	72.6	34.3	37.5
Magnesia-base Sulphite Waste Liquor (Sugar content, 2.5 per cent)					
L-1	2	1,150	75.4	52.5	
L-6	4	645	71.5	49.0	

[1] Values are averages of a series of experiments.

[2] HARRIS, E. E., M. L. HANNAN, R. R. MARQUARDT, *Paper Trade Jour.*, Nov. 27, 1947.

Propagation was carried out at 35°C. For each kilogram of yeast produced, 20 cu.m. of air were supplied.

The suspension of yeasts was centrifuged in a battery of Westphalia centrifuges of the direct-drive type. The cream thus obtained was washed by dilution with water, centrifuged again, concentrated to a 20 per cent cake with a rotary filter, and dried on Oschatz double-drum driers.

The yield of yeast was 45 to 48 per cent based on the sugar.

For the manufacture of 500 tons of yeast per month at the Regensburg plant, the following chemical materials were necessary, according to Saeman, Locke, and Dickerman:[1]

Material	Tons
Ammonium sulphate	10
Liquid ammonia	75
Potassium chloride	15
Diammonium phosphate	30
Magnesium sulphate	8
Trisodium phosphate	0.5
"Garfett"	7.5
Lipon (another antifoam)	9.5

Peterson, Snell and Frazier[2] studied approximately 150 fermentations of hydrolyzates from 13 different species of wood in which nine types of yeasts were used.

These investigators prepared the wood hydrolyzates for fermentation by four different procedures, but the following one was most successful. The amounts of the various materials used was based on 5 to 6 per cent reducing sugar. Calcium carbonate was added to the hydrolyzate until the pH was 5.0 (approximately 0.75g./5g. reducing sugar). Next, 0.05 per cent of sodium sulphite was added. The liquor was then heated to boiling with live steam, cooled and filtered by passage through a pad of Hy-Flo Supercel. The nutrient requirements were satisfied by adding 0.06 g. of urea and 0.05 g. of potassium dihydrogen phosphate for each gram of reducing sugar present. The medium was then diluted to the desired concentration.

The standard laboratory procedure for yeast propagation by Peterson and his associates was as follows: A 6,300-ml. amount of the medium was placed in a 5 gal. Pyrex bottle. It was inoculated with 700 ml. of an inoculum which contained 1 g. of cells per 100 ml. A canvas aerator was placed "well below the surface of the medium." To prevent excessive

[1] Saeman, Locke, and Dickerman, *loc. cit.*

[2] Peterson, W. H., J. F. Snell, and W. C. Frazier, *Ind. Eng. Chem.*, **37**: 30 (1945).

foaming 10 ml. of Vegifat Y were added. The Pyrex bottle was introduced into a constant-temperature bath (or incubator) at 30°C. Saturated air at the rate of 20 to 40 liters per min. was passed through the aerator into the medium. The propagation was allowed to continue for 24 hr.

Yields of 35 to 40 per cent of dry yeast, based on the total reducing sugars, were obtained from such yeasts as *T. utilis* No. 3, *Mycotorula lipolytica* P-13, and *Hansenula anomala*. Approximately 90 per cent of the apparent reducing sugar was utilized.

Production from Molasses, Sugar Juice, and Raw Cane Sugar.—Commercial production of food yeast from molasses, sugar juice, and raw cane sugar has been carried out in a plant located at Frome, Jamaica, West Indies. This project was undertaken by the Colonial Office of Great Britain as a result of the need for such a product and of the information and experience obtained at the pilot plant operated at Teddington, England, by the Department of Scientific and Industrial Research. A description of some of the progress made at Teddington and at Jamaica has been furnished by the Colonial Food Yeast, Ltd.,[1] by Thaysen,[2] and by others. The commercial process, as reported by these sources, will be briefly described.

PREPARATION OF WORT.—The wort is made up of the molasses (or sugar juice, or raw cane sugar), nutrients, and water. The molasses is diluted to a concentration just over 10 per cent carbohydrate. If sugar juice, or raw cane sugar, is used, it is similarly adjusted for concentration. It is "sterilized" by heating it to 93.3°C. (200°F.), filtered through plate and frame filters to separate out suspended solids, cooled to 37.8°C. (100°F.) by passage through a heat exchanger, and run into closed mild steel storage tanks.

Ammonium sulphate and superphosphate are dissolved, purified, and adjusted to the desired concentrations in separate equipment. Caustic soda, which is used to adjust the reaction of the fermentation medium, is separately prepared in solution form, also.

The molasses is adjusted to a 10 per cent carbohydrate concentration by dilution with sterile water in a special mixing tank. Ammonium sulphate, superphosphate, and caustic soda are mixed with the molasses in the correct proportions.

THE FERMENTORS.—The fermentors measure 62.5 in. in diameter by 24 ft. in height and are constructed of stainless steel. They are supplied with cooling coils and aeration devices. The latter consists of 32 Aerox aeration candles, each with 0.6 sq. ft. of surface, located at the bottom

[1] Colonial Food Yeast, Ltd., Food Yeast, May, 1944.

[2] THAYSEN, A. C., *Food*, p. 116, May, 1945.

of each fermentor. The pores of the candles do not exceed 10 microns. Thus, the air particles are very small and extremely numerous, increasing the efficiency of aeration. The fermentors are designed for continuous usage for 24 hr. followed by emptying and cleaning.

OPERATION.—The fermentors are initially filled half-full with dilute wort, containing about 200,000,000 yeast cells per milliliter. After about 3 hr., the rate of multiplication of the cells reaches a maximum wherein it requires approximately 100 min. for a new cell to form from a mother cell. The wort is now admitted at a rate such that the concentration of cells will be about 2,000,000,000 per ml. by the time the fermentor is full. From this time on, one-fourth of the total volume of the fermentor is replaced with the same volume of dilute wort each hour. The cell concentration remains at approximately 2,000,000,000 cells per ml.

A measuring device controls the rate at which the wort is fed to the fermentors. It may be set at a constant rate or at a logarithmically increasing rate. When the fermentors are operated on a continuous basis, another measuring device admits sufficient water to dilute the incoming wort to a concentration of 4.5 per cent sugar.

The pH of the medium is adjusted with caustic soda.

The fermented wort is withdrawn from the bottom of the fermentor by means of overflow pipes and collected in a tank.

FINAL STEPS.—The fermented wort is passed through a strainer and then to centrifuges (De Laval-DVK$_6$). It is separated into a suspension, referred to as yeast cream, and spent wort. The latter is discarded to the sewer. The cream is diluted with water and recentrifuged. The latter process is repeated. After these two washing treatments, the yeast cream is dried on single roll drum driers to a moisture content of about 5 per cent.

The dried yeast is passed under an electric magnet for the purpose of removing any tramp iron. It is then ground, packaged, and stored, or otherwise handled. Action, of course, must be taken to prevent the absorption of moisture by the dried product. The ground, dried preparation weighs 27 to 30 lb. per cu. ft.

CAPACITY OF PLANT.—This is expected to be 12 tons of dried yeast per day of 24 hr.

Production from Wood Sugar Stillage.—Kurth,[1] Kurth and Cheldelin,[2] Harris and associates,[3] and others have studied the production of fodder yeasts from wood sugar stillage on a laboratory basis. Wood sugar

[1] KURTH, E. F., *Ind. Eng. Chem.*, **38** (No. 2.): 204 (1946).
[2] KURTH, E. F., and V. H. CHELDELIN, *Ind. Eng. Chem.*, **38**: 617 (1946).
[3] HARRIS, SAEMAN, MARQUARDT, HANNAN, and ROGERS, *loc. cit.*

stillage (still waste liquor) is the product remaining after wood sugar hydrolyzates are fermented with yeasts and subsequently distilled and contain pentoses (xylose and arabinose), acids (acetic, formic, etc.), ash, and other constituents. The compositions of the waste liquor obtained from a Douglas fir hydrolyzate and the spent liquors after fermentation are shown in Table 55.

TABLE 55.—ANALYSIS OF LIQUOR BEFORE AND AFTER TORULA GROWTH[1]

	Still waste liquor	Torula spent liquors
Total solids, per cent	3.2	2.40
Reducing sugar (xylose), per cent	0.81	0.20
Reducing sugar after hydrolysis with H_2SO_4, per cent	0.97	0.34
Volatile acid (acetic), per cent	0.24	0.10
Volatile and nonvolatile acid (acetic), per cent	0.78	0.31–0.5
Ash, per cent		
Residue upon combustion	0.65	0.36–0.47
Residue converted to sulphate	1.03	0.58–0.68
Total carbon, per cent	1.42	0.94–1.04
5-day B. O. D	16,400	9,600
pH	5.0	7.5–8.0

[1] KURTH, E. F., *Ind. Eng. Chem.*, **38** (No. 2): 204 (1946).

The yeasts used by Kurth and Cheldelin[1] were *Torulopsis utilis* No. 3, *Mycotorula lipolytica* P-13, and *Hansenula suaveolens* Y-838. They were transferred repeatedly in a wood stillage medium to increase their rate of growth. As inoculums, yeast concentrations of about 100,000,000 cells per ml. were desirable.

The conditions of fermentation were as follows: The medium contained wood sugar stillage from Douglas fir, reinforced by the addition of urea and diammonium hydrogen phosphate in 0.05 per cent concentrations.[1] The pH was maintained between 5.0 and 6.5 with ammonia. After inoculation the liquor was aerated. The rate of growth of the yeast and also of the sugar consumption were affected to the largest extent by aeration. It was discovered that it was most important to use small-sized air bubbles for best results. Table 56 shows the effect of aeration on propagation time and final reducing-sugar content. With suitable aeration the assimilable sugar may be consumed in 18 hr. or less.[2] The rate of sugar consumption was increased as the temperature was increased from 24 to 34°C.

[1] KURTH, CHELDELIN, *loc. cit.*
[2] KURTH, *loc. cit.*

TABLE 56.—EFFECT OF AERATION[1]

Aerator	Time, hr.	Final reducing sugar, per cent
C-porosity fritted-glass tubes	16	0.30
	19	0.20
Cloth or EC-porosity fritted-glass disks	24	0.20–0.22
[illegible] glass tubing	24	0.40–0.49
Slotted disks	72	0.30

[1] Kurth, E. F., and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 290 (1946).

Kurth and Cheldelin[1] found that there was but little distinction among the different species of yeasts used in respect to their rate of growth, sugar utilization, yield, and nutritional value. All were able to utilize a large proportion of the residual sugars and acids in the wood sugar stillage. Yields of dry yeasts varied from 53 to 63 per cent on the basis of the sugar consumed.

Data concerning the contents of protein, amino acids, and B vitamins of the three yeasts studied by Kurth and Cheldelin are given in Tables 57 and 58.

TABLE 57.—AMINO ACIDS AND PROTEIN CONTENT OF YEASTS
(Percentage of oven-dry weights)[1]

Yeast	*Mycotorula*	*Hansenula*	*Torula*	Brewers'[2]
Total protein	51.0	53.4	52.9	51.8
Amino acid				
Arginine	3.2	2.9	3.1	2.7
Histidine	1.4	1.4	1.5	1.3
Lysine	4.4	4.3	4.4	3.5
Phenylalanine	2.4	2.4	2.3	2.4
Tryptophane	0.3	0.3	0.3	0.8
Threonine	2.5	2.4	2.5	2.8
Leucine	3.7	3.6	3.8	3.7
Isoleucine	3.5	3.7	3.7	3.1
Valine	3.1	3.3	3.3	2.4

[1] Kurth, E. F., and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 617 (1946).
[2] Reported by R. J. Block and D. Bolling, *Arch. Biochem.*, **7**: 313 (1945) for strain K 105.
[3] Values are expressed in terms of the [illegible]

The production of fodder yeast by a continuous method from wood sugar stillage has been described by Harris and associates[2] (refer to page 263).

[1] Kurth, and Cheldelin, *loc. cit.*
[2] Harris, Salman, Mardon, Hannan, Rogers, *loc. cit.*

Fodder Yeast from Citrus-waste Press Juice.—Nolte, von Loesecke, and Pulley[1] described the production of fodder yeast from citrus-waste press juice. This juice is obtained by pressing ground citrus waste (peel, rag, and seeds) that has been limed. It contains 4.08 to 8.58 per cent of total sugars with an average of 6.63 per cent. It is deficient in phosphates and nitrogen.

TABLE 58.—B VITAMIN CONTENT OF YEASTS

	Vitamin content, γ/g. dry wt.[1]		
	Mycotorula	*Hansenula*	*Torula*
Thiamine	5.3	8.5	6.2
Riboflavin	59	54	49
Nicotinic acid	600	590	500
Pantothenic acid	.....	180	130
Biotin	1.8	1.7	1.8
Folic acid	3.1	1.7	2.8
p-Aminobenzoic acid	31	16	17

[1] KURTH, E. F., and V. H. CHELDELIN, *Ind. Eng. Chem.*, **38**: 617 (1946).

The juice is prepared for use by screening it, heating it to the boiling temperature, and holding it for 5 min., adding filter aid to it and filtering, diluting it with tap water to adjust the total sugar content to 1 per cent, and cooling to 30°C. Trisodium phosphate and ammonium sulphate are added to the juice in the proportion of 9 per cent based on the total amount of sugar in the batch. Since additional ammonium sulphate is required, it is added during the process at the end of 1 hr. and during the succeeding 3 hr. at the rate of 1.75 per cent per hr. Sufficient sodium carbonate is added to maintain the pH between 4.4 and 6.5.

The nutrient citrus-waste press juice is inoculated with 4 per cent by volume of a 2-day culture of *Torulopsis utilis* in malt wort (which contained 6 per cent of total carbohydrates at the time of inoculation). The medium is aerated vigorously with filtered air and maintained at a temperature of 29 to 30°C. The process is generally completed in 8 hr. The yeast is separated by centrifuging and washed. The yields from citrus-waste juice containing 1 per cent of total sugar (after dilution) are 44.3 to 48.0 per cent of dry yeast (177 to 191.9 per cent of wet yeast containing 25 per cent of solids) based on the total amount of sugar in the juices.

[1] NOLTE, A. J., H. W. VON LOESECKE, and G. N. PULLEY, *Ind. Eng. Chem.*, **34**: 670 (1942).

Production of Fodder Yeast on a Continuous Basis.—The continuous propagation of yeast has been studied by Harris and his fellow researchers.[1] The equipment used (refer to page 267) was a modification of the Waldhof fermentor, which was used in Germany during World War II for the continuous propagation of food yeast from sulphite liquors. It consisted of an open tank of 34-liter fermentation medium capacity; a mechanically driven spinner, 6 in. in diameter, which was operated at a speed of 1,100 r.p.m. and served as a combination aerator and agitator by drawing the fermentation liquor down through a draft tube and forcing it up and around the sides; a proportioning pump to control continuous feeding; and a standpipe, which was slightly higher than the draft tube, for controlling continuous discharge.

The propagation was carried out as follows: Two liters of a solution that contained 100,000,000 yeast cells per ml. (*T. utilis* No. 3) were placed in the fermentor. The spinner was put into operation and then wood sugar hydrolyzate, diluted to a concentration of 2.5 per cent of reducing sugars and containing nutrients, was added at a rate of 2 liters per hr. Air was introduced at the rate of 0.5 cu. ft. per min. The spent medium containing the yeast started to overflow from the fermentor into the standpipe in about 5 hr. The yeast was separated from the liquor by centrifuging and returned to the fermentor until the volume of wet yeast in the fermentor was about 7 per cent, or 1.8 per cent dry yeast. At this point, undiluted wood hydrolyzate, of 4.5 to 5 per cent concentration, was admitted and the rate of air flow was increased to 0.75 cu. ft. per min. The temperature of fermentation was 30 to 32°C. and was controlled by cooling coils and an electric heater. No antifoam agent was required due to the type of aerator used. The initial pH of the fermentation medium was 4.7 to 6.6, that of the feed 4.2 to 4.5.

Results of the propagation of *T. utilis* on various types of media and under various conditions are shown in Table 59.

EFFECT OF IRON PRODUCTION OF VITAMINS.—Lewis investigated the effect of the iron nutrition of *T. utilis* on the synthesis of vitamins.[2] He found that iron deficiency caused increased rates of synthesis of thiamin, riboflavin, nicotinic acid, pyridoxin, and pyridoxin isotels per gram of yeast and decreased rates of synthesis of biotin, inositol, *p*-aminobenzoic acid, and Norit eluate factor isotels. However, no effects on the synthesis of pantothenic acid were observed.

Nutritive Value.—The nutritive value of food yeast has been studied by a large number of researchers, particularly in Germany, Great Britain, and this country. These studies have been concerned with such subjects

[1] HARRIS, SAEMAN, MARQUARDT, HANNAN, and ROGERS, *loc. cit.*
[2] LEWIS, J. C. *Arch. Biochem.*, **4** (No. 2): 217 (1944).

TABLE 59.—CONTINUOUS PROPAGATION OF *Torula utilis* ON WOOD HYDROLYZATE, ON SUGAR RESIDUES FROM ALCOHOLIC FERMENTATION OF WOOD SUGARS, AND ON SULPHITE LIQUOR[1]

Series No.	Rate of feed, liters/hr.	Sugar concentration, per cent	pH	Air, cu. ft./min.	Air, cu. ft./lb. of yeast	Reducing sugar used, per cent	Dry-yeast yield based on total sugar, per cent	Protein content of yeast, per cent
			Wood hydrolyzate					
9	3.0	4.5	4.7	0.75	415	88.4	36.4	52.0
10	2.0	4.5	5.0	0.75	630	92.0	35.9	52.3
11	4.0	4.5	5.0	0.75	313	86.5	36.0	52.7
12	4.5	4.5	5.0	0.75	275	85.3	36.5	54.4
13	5.0	4.5	5.0	0.75	296	83.1	30.5	52.5
14	3.0	4.6	6.6	0.75	295	93.8	49.7	58.6
15	4.0	4.6	6.0	0.75	210	93.4	52.6	57.3
16	4.5	4.6	6.0	0.75	212	93.2	46.5	56.5
17	5.0	4.6	6.0	0.75	203	91.1	43.4	52.5
18	5.0	4.6	5.5	0.75	208	90.4	42.5	51.9
			Fermentation residues					
19	3.0	0.94	5.4	0.60	1100	60.0	52.0*	50.0
20	4.0	0.91	5.5	0.60	930	55.0	47.0*	50.0
			Sulphite liquor					
21	2.0	1.66	5.0	0.50	1040	79.0	39.2	52.7
22	3.0	1.66	5.0	0.50	695	78.0	39.0	52.5
23	4.0	1.66	5.0	0.50	540	75.0	37.6	52.0
24	5.0	1.66	5.0	0.50	550	59.0	29.6	51.0

[1] HARRIS, E. E., J. F. SAEMAN, R. R. MARQUARDT, M. L. HANNAN, and S. C. ROGERS, *Ind. Eng. Chem.*, **40** (No. 7): 1220 (1948).

* Values corrected to product with 50 per cent protein.

as the digestibility of the yeast in human nutrition; its vitamin content when grown on different media; its protein content; its amino acid content; its value as a food constituent for children and adults and as a feed supplement for hogs, cattle, chickens, rats, and other animals.

Among the reports on these subjects may be mentioned those of Fingerling and Honcamp (1933); Bunger and associates (1934); Fink and Just (1938, 1939); Scheunert and Wagner (1940); Stephenson, Penton, and Korenchevsky (1941); Dirr (1942); Dirr and Von Soden (1942); Hock (1942); Bickel (1942); Thaysen (1943); Thaysen and Morris (1943); Lewis (1944); Lewis, Stubbs, and Noble (1944); Butschek (1944); Wilson

(1944); Colonial Food Yeast, Ltd. (1944); Klose and Fevold (1945); Peters (1945); Kurth and Cheldelin (1946); Agarwal, Singh, King, and Peterson (1947). The subject was reviewed by Carter and Phillips in 1944.

The high protein content of dry yeast has already been referred to (Table 53). There is some indication that yeast protein may be deficient in the amino acid methionine and possibly in cystine, according to Hock and Fink.[1] Klose and Fevold[2] found that torula yeast and brewers' yeast contained inadequate quantities of methionine for the optimum growth rate of chicks. However, a large percentage of animal protein could be replaced satisfactorily with yeast. In spite of these indications, food yeasts may be regarded as valuable protein supplements.

Among the vitamins of the B-complex found in yeast are thiamin, riboflavin, niacin (nicotinic acid), pantothenic acid, biotin, pyridoxine, folic acid, and *p*-aminobenzoic acid. The thiamin, riboflavin, niacin, and folic acid contents of four different species of yeasts, grown in molasses media and subsequently dried, are shown in Table 60.

TABLE 60.—VITAMIN CONTENT OF YEASTS (γ/G. OF DRY YEAST)[1]

Molasses	*S. cerevisiae*				*T. utilis*				*C. arborea*				*O. lactis*			
	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid
Lansing (beet)	37.6	43.8	414.3	21.6	37.5	54 2	520.6	15.2	32.7	69.5	503.1	14.8	20.1	55.0	192.8	7.7
Mason City (beet)	35.7	50.4	443.3	21.4	36 7	62.0	600.0	10.6	33.1	52.3	492.3	16.0	28.9	39.9	212.6	5.6
Ovid (beet)	32.7	45.4	442.8	19.6	38.1	54.8	511.3	11.7	31.3	53.0	512.2	17.6	27.2	42.6	247.5	7.6
Hawaiian (cane blackstrap)	40.8	49.1	568.1	19.1	35.4	58.6	531.4	10.7	33.1	60.0	580.2	15.0	29.0	43.0	242.4	7.8

The media used for the growth of the yeasts were comprised of treated molasses (1 per cent sugar), 0.1 per cent KH_2PO_4, 0.1 per cent urea, and 2 ml. corn steep liquor. The fermentation was run for 36 hr. at 30°C.

[1] AGARWAL, P. N., K. SINGH, P. S. KING, and W. H. PETERSON, *Arch. Biochem.*, **14** (Nos. 1 and 2): 105 (1947).

A number of studies have been made on the vitamin content of *T. utilis*. Lewis and his associates[3] found 18 micrograms of thiamin, 36 micrograms of riboflavin, and 610 micrograms of niacin per gram of yeast (dry basis) produced in molasses media; and 22 micrograms of thiamin, 44 micrograms of riboflavin, and 535 micrograms of niacin per gram of yeast

[1] HOCK, A., and H. FINK, *Zeit. physiol. Chem.*, **279**: 187 (1943).
[2] KLOSE, A. A., and H. L. FEVOLD, *Jour. Nutrition*, **29**: 421 (1945).
[3] LEWIS, J. C., J. J. STUBBS, and W. M. NOBLE, *Arch. Biochem.*, **4** (No. 3): 389 (1944).

(dry basis) produced in fruit juice media. Kurth and Cheldelin[1] found 6.2 micrograms of thiamin, 49 micrograms of riboflavin, 500 micrograms of niacin, and 2.8 micrograms of folic acid per g. (on a dry basis) in yeast grown in wood sugar stillage. Thaysen[2] reported 23 micrograms of thiamin, 54 micrograms of riboflavin, and 440 to 490 micrograms of niacin per gram of yeast (dry basis) grown in molasses.

Processing of Brewers' Yeast for Use as Food.—Brewers' yeast must be debittered and otherwise treated before use as a food constituent. Reports concerning the processing of brewers' yeast have been presented by Siebel, Weber, and Singruen[3]; Haffenreffer[4]; Burton[5]; MacDonough and Haffenreffer,[6] and others.

In the process described by MacDonough and Haffenreffer,[6] yeast cream (10 per cent yeast on a dry basis) is pumped through a filter into a weighing tank where it is weighed prior to transfer to a debittering tank. The yeast is washed first with water, 10,000 lb. of water being added to 5,000 lb. of yeast slurry. The mixture is separated into a yeast concentrate and waste liquor by passage through an Alpha Laval yeast separator. The yeast is washed next with a caustic soda solution at a pH of about 12.1. Two parts by weight of alkaline water are employed for each part of yeast. The mixture is passed through the separator. The yeast is now washed with a solution containing 0.05 per cent caustic soda (5 lb. of sodium hydroxide in 10,000 lb. of water) and the mixture passed through the separator. Finally the yeast is washed with cold water and concentrated by passage through the separator.

After the debittering treatment, table salt is added to the concentrate in the amount of 2 per cent of the dry weight of the yeast. Likewise sufficient U.S.P. phosphoric acid is added to lower the pH to 5.5 to 5.7.

The yeast preparation may be enriched, if desired, by the addition of a solution containing thiamine, riboflavin, and niacin. The concentrate is stirred uniformly and then dried on a double-drum drier. The final product is pulverized and packaged.

In order to prevent vitamin losses during processing it is essential to use speed and temperatures not above 40°F. These precautions are especially important during the debittering process.

[1] Kurth, E. F., and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 617 (1946).

[2] Thaysen, A. C., Part III, Food Yeast, *Colonial Food Yeast*, Ltd., May, 1944.

[3] Siebel, R. V., P. J. F. Weber, and E. Singruen, *Modern Brewery Age*, **26** (No. 5): 44 (1941); **26** (No. 6): 46 (1941); **27** (No. 1): 49 (1942); and **27** (No. 2): 74 (1942).

[4] Haffenreffer, T. C., Jr., *Brewers Bull.*, **36** (No. 87): 4 (1943).

[5] Burton, L. V., *Food Inds.*, **15** (No. 11): 66 (1943).

[6] MacDonough, J. V., and T. C. Haffenreffer, Jr., *Wallerstein Labs. Communs.*, **7** (No. 20): 39 (1944).

LABORATORY FERMENTORS FOR YEAST PROPAGATION

A number of laboratory-scale fermentors have been developed for the propagation of yeast. One of these is illustrated in Fig. 37. Another has been described by Feustel and Humfeld.[1] Fermentors designed and

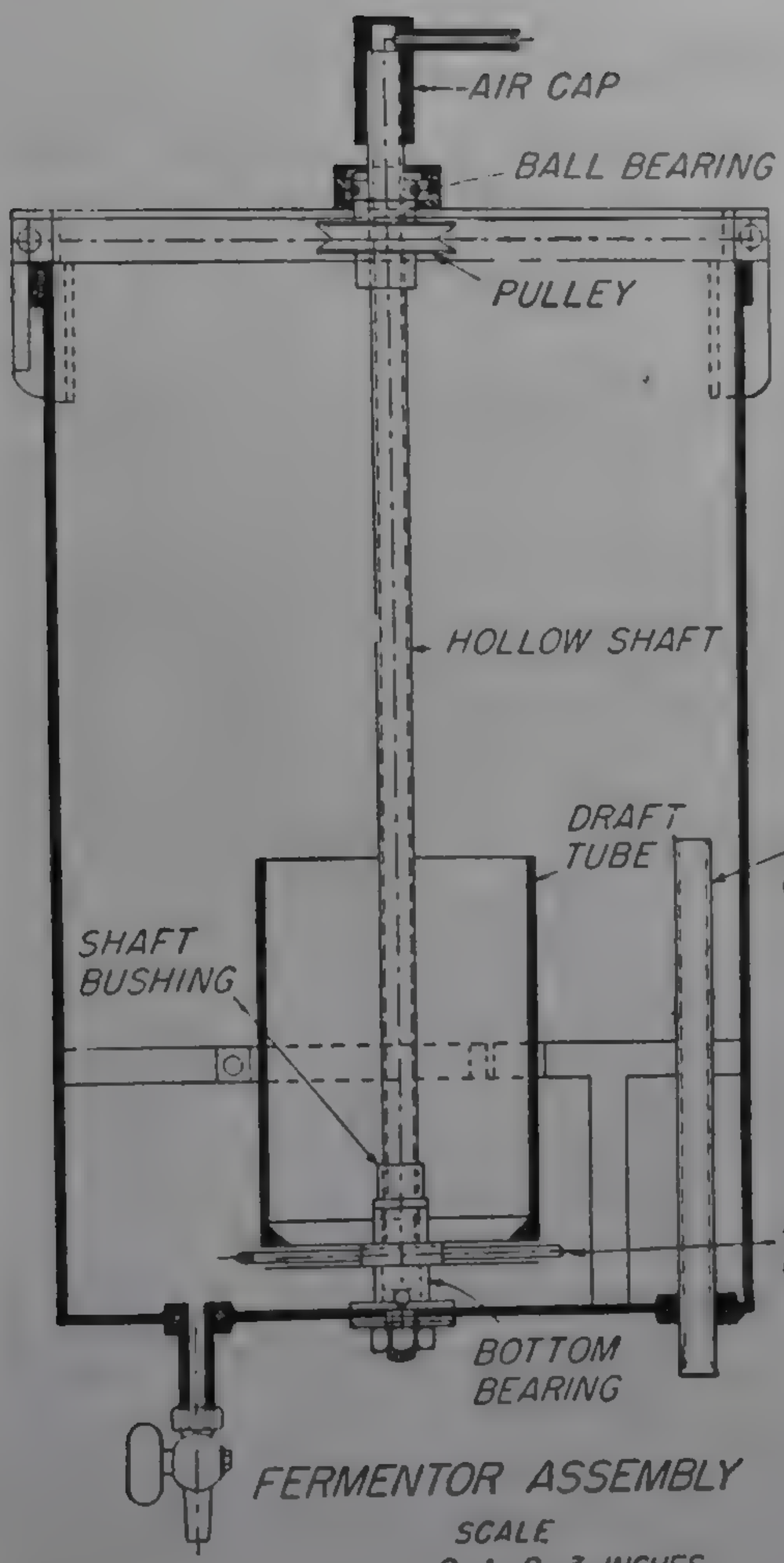
FIG. 39.—Temperature-control equipment of laboratory-scale fermentor designed and built at Forest Products Laboratory. (*Courtesy of E. E. Harris, M. L. Hannan, and R. R. Marquardt, Paper Trade Jour.*, Nov. 27, 1947.)

constructed at the Forest Products Laboratory and at the Western Regional Research Laboratory, respectively, are described below:

Forest Products Laboratory Yeast Propagator.—Harris, Hannan, and Marquardt[2] constructed a laboratory-scale fermentor (propagator) for the study of yeast production from wood sugars. Some details of the construction of this fermentor are shown in Figs. 39 and 40. The fermentor is constructed preferably of stainless steel in order to resist corrosion and lend itself to easy cleansing.

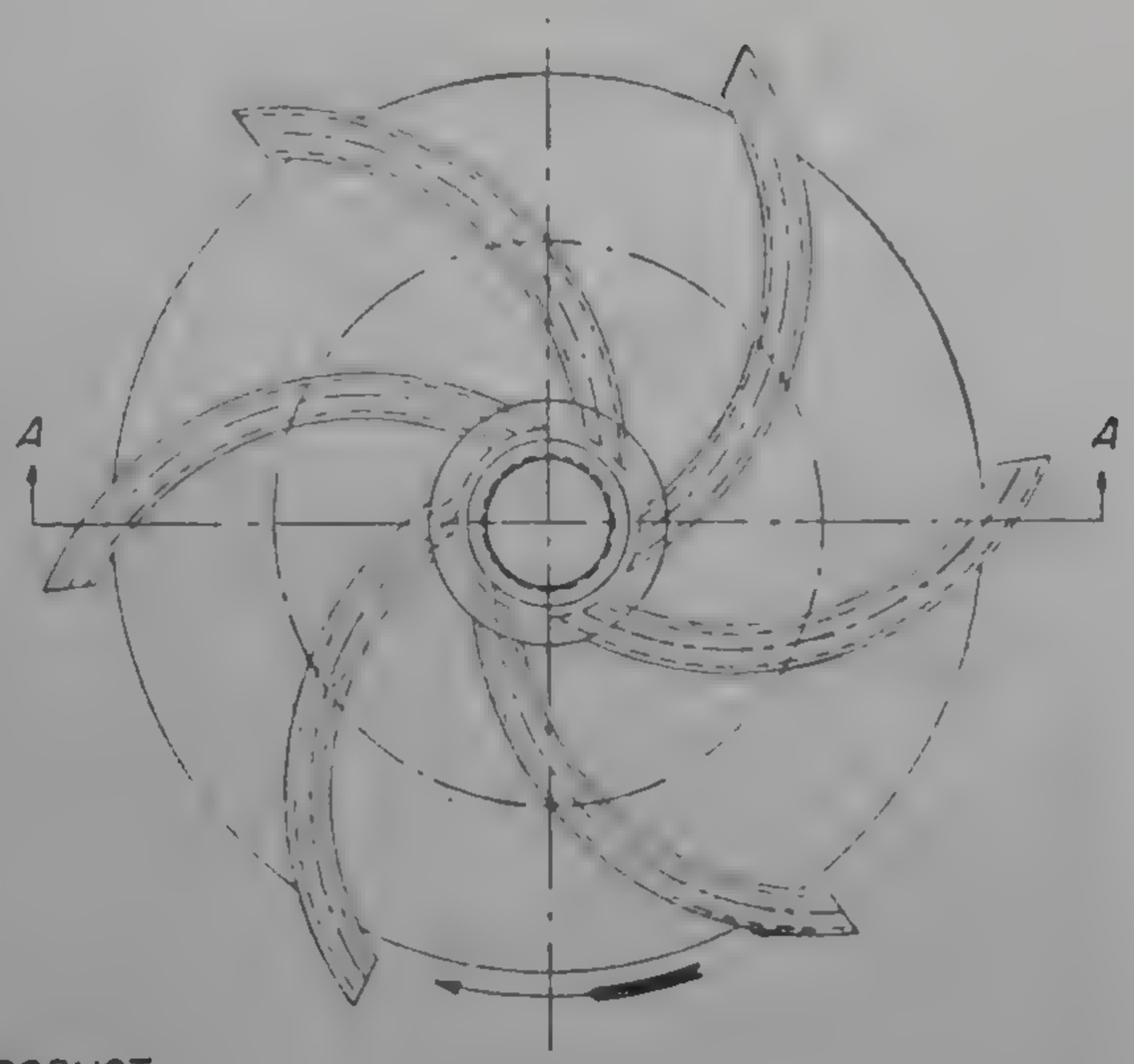
The propagator (fermentor) consists of several essential parts: an open

[1] FEUSTEL, I. C., and H. HUMFELD, *Jour. Bact.*, **52** (No. 2): 229 (1946).
[2] HARRIS, HANNAN, MARQUARDT, *loc. cit.*

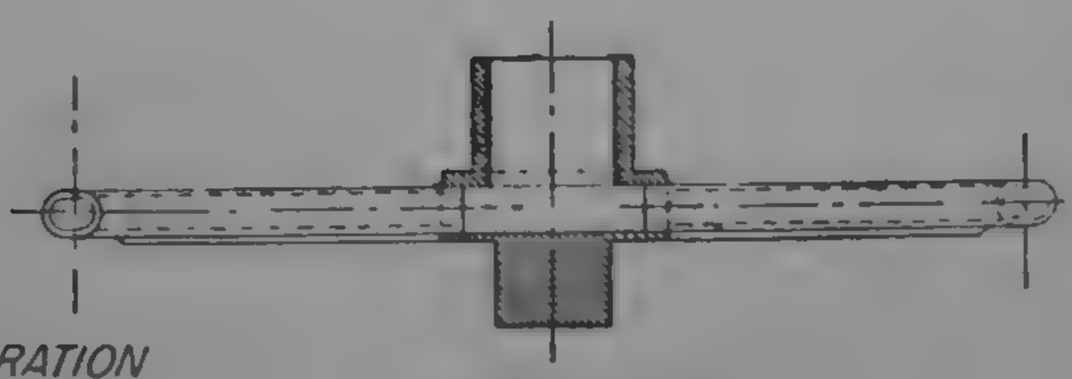


FERMENTOR ASSEMBLY

SCALE
0 1 2 3 INCHES



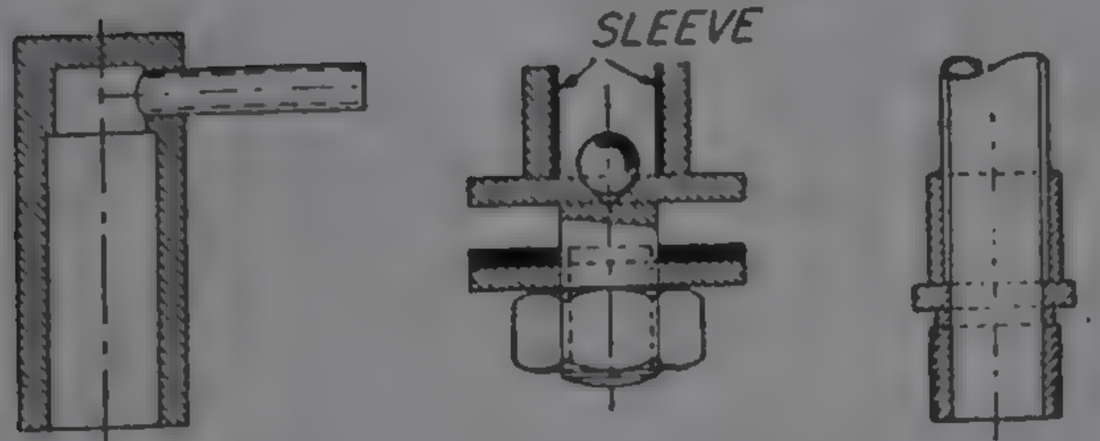
TOP VIEW



SECTION A-A

AERATION WHEEL

SCALE
0 1 INCH



AIR CAP
SCALE
0 1 INCH

BOTTOM BEARING
SCALE
0 1 INCH

SHAFT BUSHING
SCALE
0 1 INCH

Fig. 40.--Laboratory-scale experimental fermentor with mechanical aerator built at the Forest Products Laboratory with design altered somewhat from industrial-scale equipment used in Germany. [*Courtesy of J. F. Saeman, Anal. Chem.*, **19**: 913 (1947).]

tank of 34-liter capacity, with reference to the top of the overflow tube; a motor-driven proportioning pump; an aeration wheel which performs the dual functions of aerating and defoaming; a draft tube; temperature regulating equipment; and a pH controller.

During operation, the fermentation solution is fed continuously by

a proportioning pump into the top of the fermentor at a point next to the shaft of the aeration wheel. A disk on the shaft "just above the liquid" distributes it uniformly in the fermentor. Air enters the hollow shaft of the aeration wheel (which is 6 in. in diameter) at the rate of 1 cu. ft. per min. for each cubic foot of medium and is delivered to the medium through six tubes. The aeration wheel (or spinner) which revolves at the rate of 1,000 r.p.m. pulls the medium out of the draft tube and forces it up the outside liberating the gases as it drops down the draft tube.

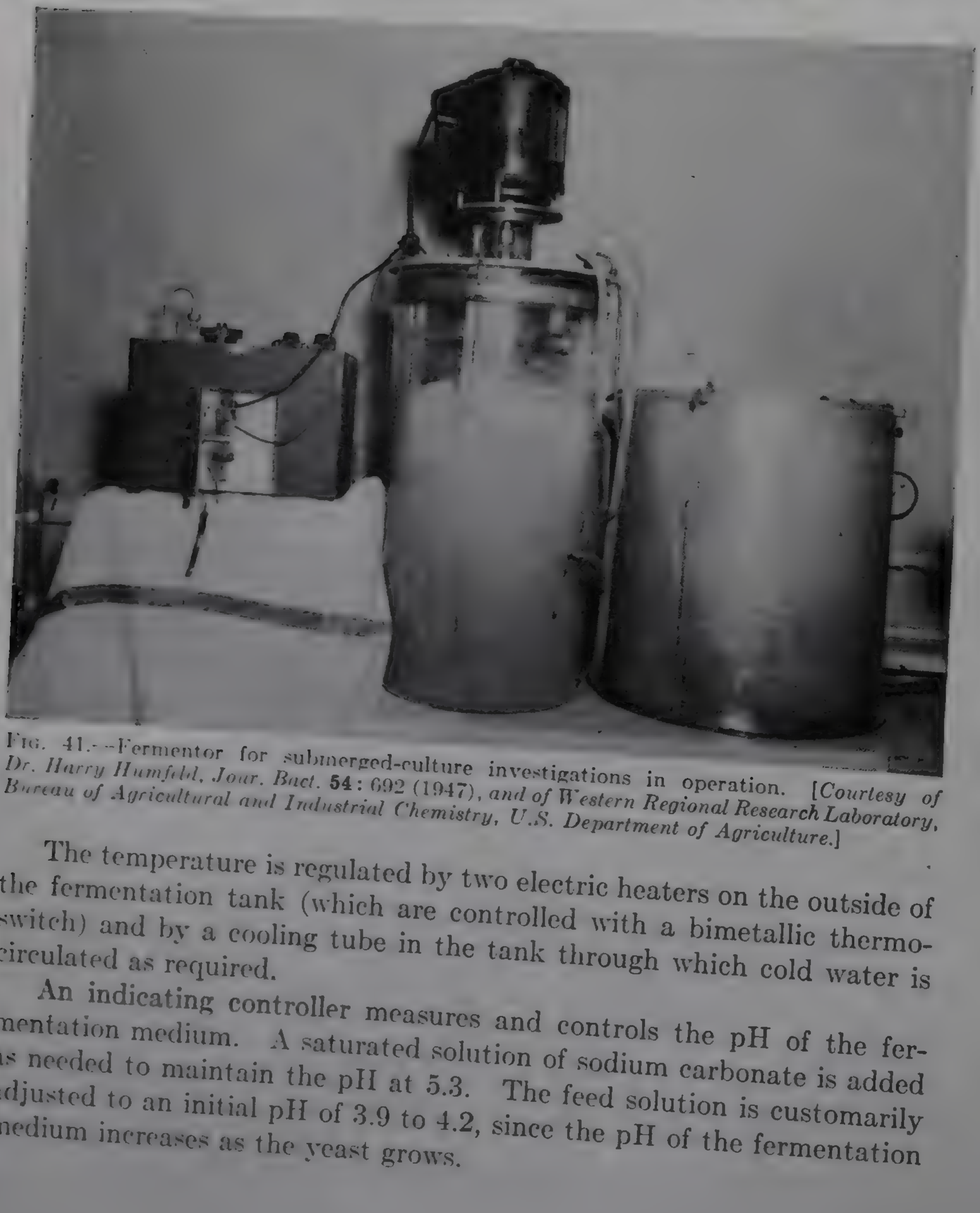


FIG. 41.—Fermentor for submerged-culture investigations in operation. [*Courtesy of Dr. Harry Humfeld, Jour. Bact.* **54**: 692 (1947), *and of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture.*]

The temperature is regulated by two electric heaters on the outside of the fermentation tank (which are controlled with a bimetallic thermo-switch) and by a cooling tube in the tank through which cold water is circulated as required.

An indicating controller measures and controls the pH of the fermentation medium. A saturated solution of sodium carbonate is added as needed to maintain the pH at 5.3. The feed solution is customarily adjusted to an initial pH of 3.9 to 4.2, since the pH of the fermentation medium increases as the yeast grows.

The yeast and spent liquor pass out through a standpipe.[1]

Western Regional Research Laboratory Fermentor.—Humfeld (1947) described an improved laboratory-scale fermentor for submerged-culture studies. This fermentor has a mechanical foam breaker and can draw air in by the suction produced by the stirring blades. It has a capacity for 10 to 18 liters of medium.

FIG. 42.—Fermentor assembly. [*Courtesy of H. Humfeld, Jour. Bact.*, **54**: 689 (1947), *and of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture.*]

The tank is a Pyrex glass jar of 12-in. diameter and 24-in. height (Fig. 41). A gasketed stainless-steel cover or lid (2) fits on the top. From this cover an agitation-aeration assembly of stainless steel (Fig. 42) is suspended. Plans for the fermentor are presented in Figs. 43 to 46. As will be seen by reference to Fig. 43, the fermentor assembly consists of an air-dispersing unit mounted at the bottom end of the stirring shaft (3); adjustable truncated cones which govern the stirring characteristics

[1] *Ibid.*

to some extent; four metal struts attached to the cover at right angles and connected at the bottom by a metal web to furnish support for the stirring shaft.

The device for aeration and agitation is located at the lower end of the shaft 3). It rotates between stationary circular plates (23 and 24). The upper one (23) is attached to bars (19 and 20); the bottom one is held to the upper plate by studs. The culture medium enters the lower plate through a circular hole (25). Four tubes (28) are attached to the shaft and form arcs at right angles to it. Vanes (27) are attached to alternate sets of the tubes (Figs. 43 and 44).

Air enters through a pipe (21), which extends from the cover to a location directly below the central core of the agitation-aeration device. When the shaft is rotated, the vanes (27) cause the medium to flow up through the circular opening (25), bore (30), tubes (28), and out of the casing and past pins (26). The medium also flows through the opening to the casing's interior and out by the pins. As the medium passes the tip of the pipe, air is sucked through the pipe into it.

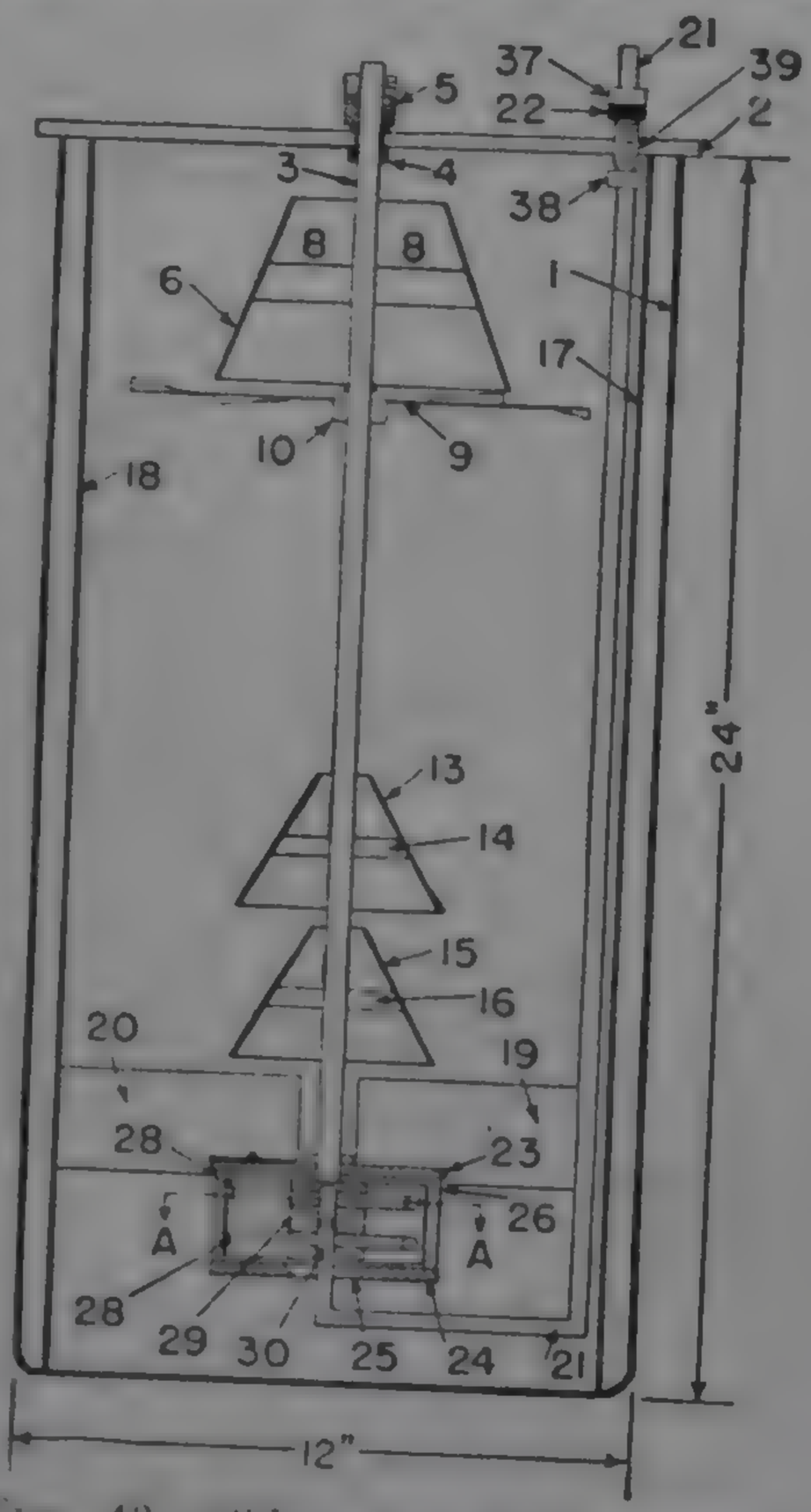


FIG. 43.—Side view of fermentor unit. (*Courtesy of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture.*)

The air passes through bore (30),

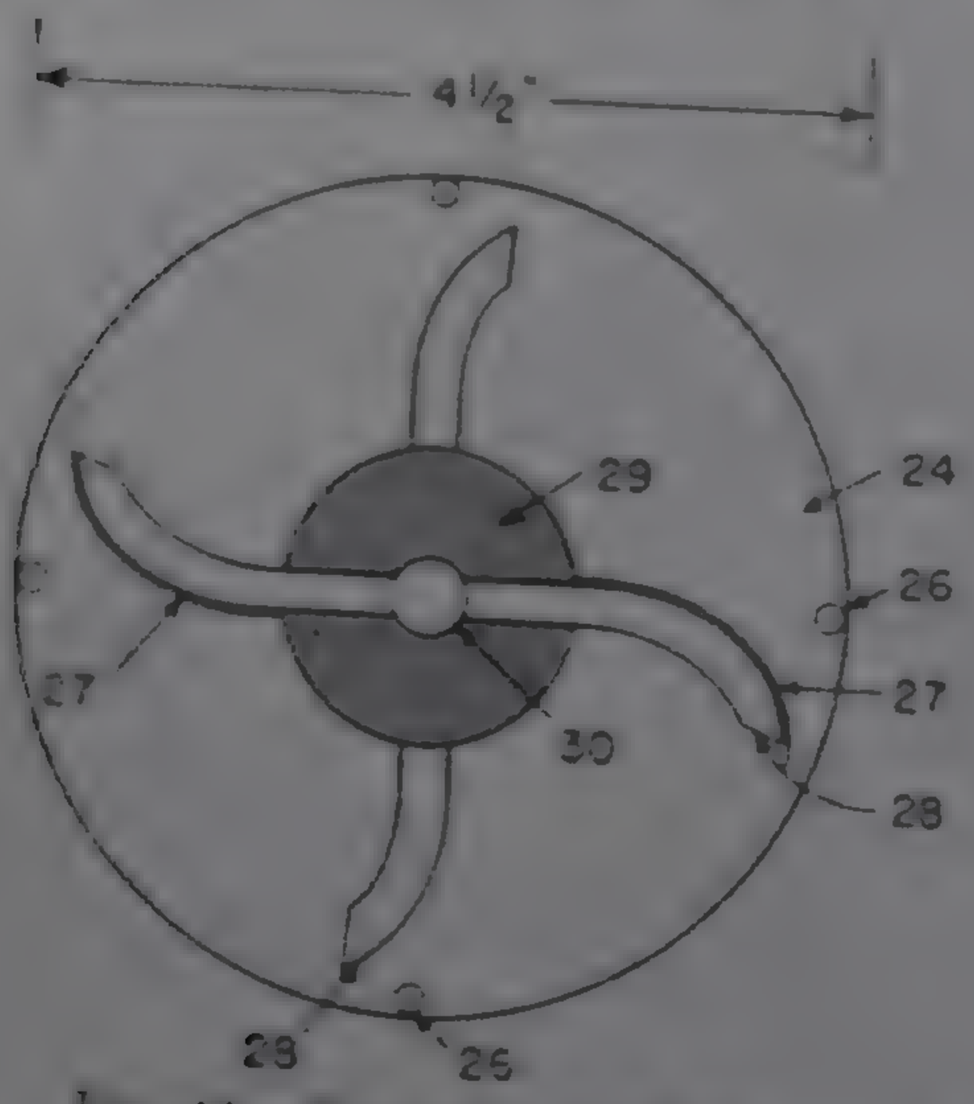


FIG. 44.—Top view of agitator.

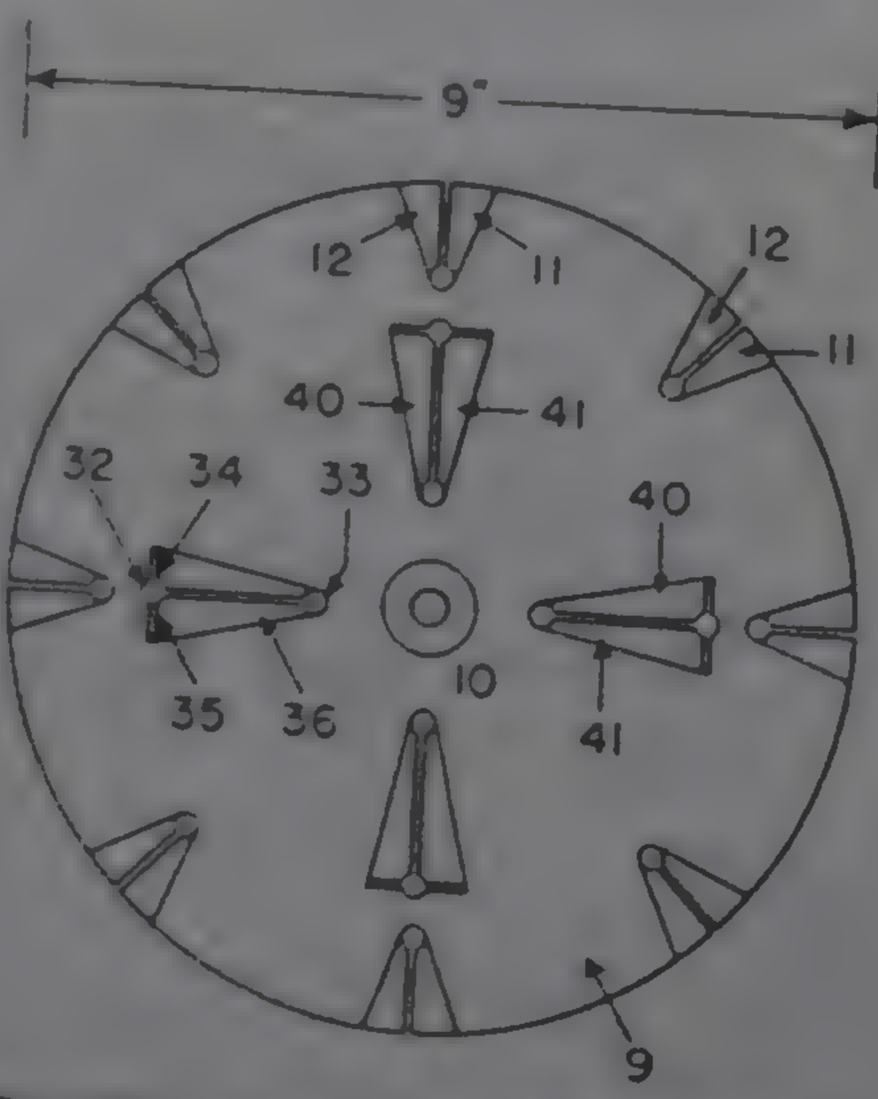


FIG. 45.—Top view of foam-breaker disc.

tubes (28), and out of the casing. The amount of air used is regulated by raising or lowering the tip of the pipe (21) in respect to the inlet of bore (30).

Cones (13 and 15) are fastened to the shaft by means of webs (14 and 16, respectively) and when rotated mix and agitate the medium.

The foam breaker consists of a cone (6) and a disc (9), located above the normal level of the medium. The disc (9) is secured to the shaft by a collar (10) and contains an interior set of vanes (40 and 41) (Figs. 43 and 44) half of which (40) are turned up and half of which (41) are turned down, and an exterior set of vanes (11 and 12), half of which (11) are turned up and half of which (12) are turned down. When the disc (9) is rotated counterclockwise, the inner vanes (40 and 41) force the medium towards cone (6) and the outer vanes tend to force it towards the side of the vessel or its contents. Thus, the foam is broken effectively. A recent modification of this fermentor consists of a change in the design of the stirrer (Fig. 44), to give a greater capacity for aeration, and the elimination of supporting struts.

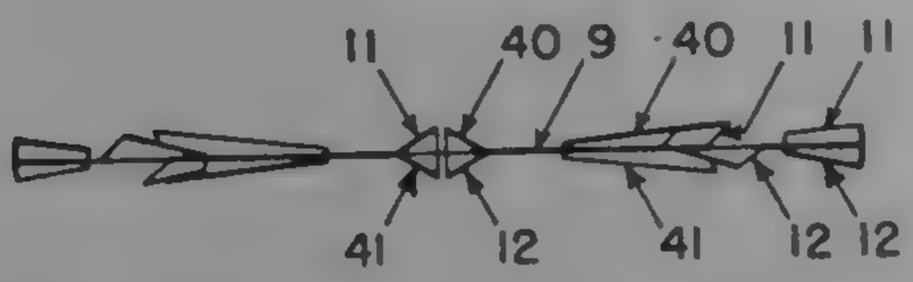


FIG. 46.—Side view of foam-breaker disc. (*Courtesy of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture.*)

The fermentor is provided with a set of pH meter electrodes and with leads protected by removable waterproof tubes. It is also provided with a thermometer well and a sampling tube. The power for operating the fermentor is supplied by a 0.25-hp., ball-bearing, variable-speed electric motor, mounted vertically.

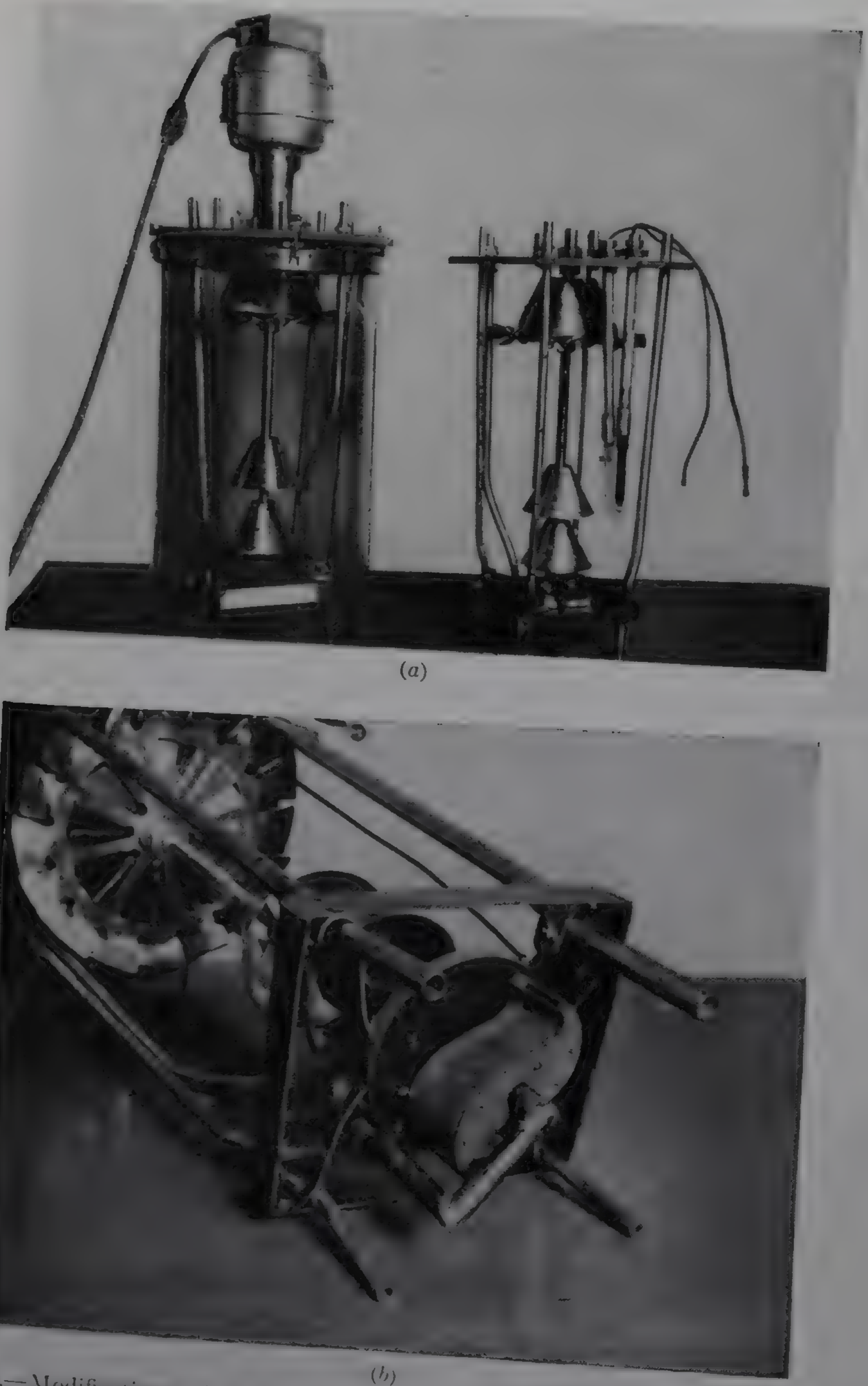
For further details of construction refer to the description furnished by the Western Regional Research Laboratory, U.S. Department of Agriculture, Albany, Calif.; for additional information on its operation refer to the paper by Humfeld.[1]

FAT PRODUCTION BY YEASTS AND YEAST-LIKE MICROORGANISMS

Only under conditions of a national emergency would one ordinarily attempt to produce fat from microorganisms, for normally the demands of a country may be met by domestic production from animals and higher plants, or by importations. But when the usual supply becomes inadequate, waste carbohydrate materials may be used for the synthesis of fat by microorganisms.

Fat may be produced by a number of different microorganisms: by yeasts; by yeast-like microorganisms, such as *Oösporα* (*Oïdium*) *lactis;* and by molds (a discussion of fat production by molds appears in Chap. XXXIV).

[1] HUMFELD, H., *Jour. Bact.*, **54** (No. 6): 689 (1947).



(*a*)

(*b*)

FIG. 46A.—Modifications of the fermentor. *a* (left), fermentor ready for operation; (right), operating unit removed from container. *b*, close-up view of operating unit of fermentor showing lower end of air-intake pipe leading into center of agitator. Two of the tubular supports serve as sampling pipes, one as a harvesting tube, and one as a thermometer well. (*Courtesy of Dr. Harry Humfeld and the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture*, 1948.)

"Fat" Defined.—The term "fat," as used in this chapter, refers to substances insoluble in water but soluble in ether and other fat solvents. In the literature, one finds that some authors use the term "lipoid" to designate the crude fat obtained from microorganisms. However, in view of the wide use of the word "fat," which is more properly restricted to mean the esters formed by combinations of fatty acids with glycerol, this term will be used throughout the present discussion to include what actually would be classified as lipids, according to Bloor.[1]

Under the term "lipid,"[1] Bloor includes the simple lipids—the fats, oils, and waxes; the compound lipids, such as the phospholipids; and the derived lipids, such as fatty acids, sterols, and alcohols.

The Fat of Yeast.—Some of the first studies concerning the nature of yeast fat were carried out by Nägeli and Loew (1878), Gérard (1895), Gérard and Darexy (1897), Hinsberg and Roos (1903, 1904) and Sedlmayer (1903). In recent years, amongst others, Smedley-MacLean and coworkers have made notable contributions to the knowledge of this subject.

In yeast fat are found palmitic, oleic, linoleic, and lauric acids; the phospholipids, lecithin and cephalin; ergosterol and zymosterol; and other components. A large proportion of the crude fat is unsaponifiable matter.

Production of Fat by Yeast.—Nägeli and Loew first proved that carbohydrates could be transformed to fat by yeast.[2]

Fat production is a normal function of yeast cells, but the rate of its formation and the quantity of it stored may be increased through the use of certain optimum conditions. Factors of importance in fat production are the use of a medium rich in carbohydrate, oxygenation, and phosphates.

Oxygenation of a medium is essential if greater than normal quantities of fat are desired. Nägeli in 1878 showed that the fat content of yeasts and molds could be increased from 5 to 12 per cent when they were grown in well-oxygenated media containing an abundance of carbohydrates and a scarcity of nitrogen.

The addition of alkaline phosphates (Na_2HPO_4 and KH_2PO_4) to a well-oxygenated medium containing 4 per cent glucose caused increased fat storage in a suspension of brewery yeast.[3]

Usually the rate of fat formation was greatest at the beginning of the experiment, diminishing later. When the concentration of sugar was

[1] Bull, H. B., "The Biochemistry of Lipids," John Wiley & Sons, Inc., New York, 1937.

[2] Nägeli, C., and O. Loew, *Ann.*, **193**: 322 (1878).

[3] Smedley-MacLean, I., and D. Hoffert: *Biochem. Jour.*, **18**: 1273 (1924).

increased, the amount of phosphate absorbed by the yeast cells was likewise augmented.

It has been demonstrated that oxygenating a solution containing a suitable sugar increases the amount of fat stored in the suspended yeast. If yeast containing reserve carbohydrate is suspended in oxygenated water, part of the carbohydrate is converted into fat.[1]

Although investigations have been carried out using oxygenated solutions of various simple carbon compounds, thus far only ethyl alcohol and sodium acetate, two-carbon compounds, have produced significant increases in the amount of fat stored by the suspended yeast.[2] These compounds must be used in relatively dilute solution (a 0.5 to 0.6 per cent concentration of ethyl alcohol is satisfactory), because increasing the concentration of either above a low maximum leads to poor results.

The rate at which oxygen is supplied to solutions of ethyl alcohol and sodium acetate determined chiefly the amounts of lipids stored in yeast. When a solution of these compounds is not oxygenated the storage of fat and carbohydrate is not increased.

Alcohol vapor, in the presence of oxygen, leads to a deposition of lipoid material in the cells of brewery yeast and also in *Endomyces vernalis*, according to the researches of Lindner and Unger.[3] Yeast (Frohberg type), when grown in agar in a chamber containing the vapors of ethyl alcohol and oxygen, showed definite increases in fat content.[4] By keeping the moisture content of the yeast low through the use of a drying atmosphere, the highest yields of fat were obtained in the presence of alcohol vapor. Substitution of ethyl alcohol with propyl alcohol in control experiments yielded negative results.

The following table indicates the average increases in the fat content of samples of yeast incubated in oxygenated water and in oxygenated solutions of N/11 acetates.[5]

TABLE 61.—AVERAGE INCREASES IN FAT CONTENT OF YEAST IN VARIOUS SOLUTIONS

Solution	Percentage Increase in Fat Content
Water	41
Potassium acetate	180
Sodium acetate	160
Magnesium acetate	118
Calcium acetate	100

[1] SMEDLEY-MACLEAN, I., *Ergeb. Enzymforsch.*, **5**: 285 (1936).

[2] SMEDLEY-MACLEAN and HOPKINS, *op. cit.*, **20**: 343 (1926).

[3] LINDNER, P., and UNGER, *Wochschr. Brau.*, **36**: 188 (1919); LINDNER, P., *Zeit. Phys. Chem.*, **35**: 110 (1922).

[4] HALTER, H., *Biochem. Zeit.*, **225**: 249 (1934).

[5] MACLEAN, I. D., and I. SMEDLEY-MACLEAN, *Biochem. Jour.*, **32**: 1571 (1938).

When phosphate is added to a 0.6 per cent solution of sodium acetate, there is no increase in the quantity of fat produced from the acetate.

Fat is produced from sodium lactate and sodium pyruvate in oxygenated solutions containing yeast, according to Smedley-MacLean and coworkers. Shaking the solutions of these compounds increased the quantity of fat and carbohydrate stored. Lactic acid, or lactate, usually yields carbon dioxide and small amounts of volatile acids in addition to the stored products. Pyruvate is not so effective as lactate in forming storage compounds.

No increases in the lipid content of yeast, not equally well obtained by oxygenating a suspension of yeast in water, were observed by oxygenating solutions of the sodium salts of citric, crotonic, fumaric, gluconic, levulic, maleic, or succinic acids; acetoin; 2:3-butyleneglycol; or methyl-ethyl ketone.[1]

The addition of calcium or magnesium ions to an oxygenated solution of glucose significantly decreased the quantity of lipids usually stored in yeast. Likewise, the addition of these ions to oxygenated solutions of acetates caused a decrease in the amount of lipid material ordinarily formed from the acetates.[1]

Fat Production by Endomyces Vernalis.—Considerable research was undertaken by Lindner and his associates at the Institut für Gärungsgewerbe of Berlin at the time of World War I in an effort to produce fat from *Endomyces vernalis* on a successful economic basis.[2]

E. vernalis grows as a mat or skin over the surface of a liquid medium. For fat production, oxygen is essential. However, agitation of the medium is not beneficial.

Several carbohydrates are assimilated but not fermented. Since fat storage is sought, this is a desirable feature of the organism. Molasses, cellulose waste, hydrolyzed wood, and other media containing an assimilable source of carbohydrate may be used as raw materials for fat production.

Waste sulphite liquor, fortified with nitrogenous substances and the necessary salts, is a favorable raw material. Nitrogen-containing substances may include yeast water, ammonium salts, urea, urine, molasses slop, extracts of grains, or other products. Potassium chloride, primary potassium phosphate, and magnesium sulphate may be used as salts.

An abundance of a suitable carbohydrate is essential for the maximum production of fat.

The optimum temperature for growth is 15 to 20°C., although a temperature as low as 10°C. may be used.

[1] *Ibid.*

[2] FINK, H., H. HAEHN, und W. HOERBURGER, *Chem. Ztg.*, **61**: 689, 723, 744 (1937).

In the production of fat by *E. vernalis*, two phases may be recognized in the incubation period: the phase during which the principal growth of the organism occurs (2 to 3 days under optimum conditions) and the phase of principal fat formation, which generally requires 6 to 8 days in addition. The phase of growth has been designated as the "protein generation" by Lindner. During this phase, the organism may be satisfactorily used for inoculation purposes. For growth the organism may be cultivated in a medium rich in nitrogen but poor in carbohydrates, but for fat production ("fat generation") a medium rich in a carbohydrate is essential. Thus is illustrated the difference between the optimum conditions for growth and optimum conditions for the production of a desirable end product.

Though many efforts were made to find satisfactory methods for producing fat from *E. vernalis*, only two methods were carried out on a large scale: the floor process and the pan process.

1. *The Floor Process.*—In this process, inert materials such as chopped straw or coarse sawdust were washed, impregnated with the nutrient medium, and sterilized. The impregnated inert material was spread in thin loose heaps over the floor and inoculated with a fine suspension of *E. vernalis*. The heaps were turned several times a day and occasionally sprayed with water. At 11°C., fat production was completed in approximately 12 days; at 20°C., in 8 to 10 days. The fungus was dried at a temperature of not above 50°C.

The following data (Lindner) are taken from a test in which the inert material was impregnated with a 25 per cent molasses solution and inoculated with *E. vernalis:*

TABLE 62.—DATA ON FAT PRODUCTION BY *Endomyces vernalis*

Amount of sugar used	12.5 kg.
Sugar used in CO_2 production	4.16 kg.
Sugar left for fat formation	8.34 kg.
Theoretical yield of fat	3.33 kg.
Actual yield of fat	0.95 kg.
Percentage of theoretical yield	28.5
Yield on basis of sugar used, per cent	7.6

The amount of sugar converted to fat by this process was small. Furthermore, difficulties were encountered in separating the fungus from the inert material, frequent attention was necessary during growth of the organism, and infection, especially with *Torula*, was prevalent. There was, however, adequate surface exposure.

2. *The Pan Process.*—In this process, which was carried out on a large scale, the organism was grown in large flat pans containing a shallow layer (1 to 2 cm. in depth) of a sterilized nutrient solution of sugar.

In order to conserve space and to facilitate handling, the pans were placed one above the other in frames. No covers were placed over the pans.

When the mat, or growth, on the surface of the solution in the pan became well-developed, a large part of the culture solution was carefully run off and replaced with fresh nutrient sugar solution. After the maximum fat formation (usually in 7 to 8 days), the spent culture solution was drawn off and the mat carefully washed by repeated underlayerings with water. The mats thus obtained, rich in fat, were used as a paste. Such paste was designated, for example, as "Evernal" or "Myceta." The protein content of such pastes was valuable.

In this process sugar utilization was nearly complete, but the necessary use of much hand labor increased the costs, while infection by yeasts, molds, or bacteria was a very serious matter in some cases.

Recovery of Fat.—Fat may be obtained from *E. vernalis* by one of several methods: by chemical means, by extraction with ether, and by an autolytic process. In the chemical method the fungus mat is treated with warm dilute hydrochloric acid to decompose the cells. The fat, obtained as neutral fat, may be used for food. The efficiency of the process is high.

The cells are ground with sand to disintegrate them before extraction with ether. Fat so obtained is used industrially. The efficiency of this process is not high.

Self-digestion is permitted at approximately 50°C. for 2 to 3 days in the autolytic process. The fat is recovered from the autolyzate.

Preservation of the Fat.—The fat keeps well if oxygen is completely excluded.

Fat Production by Oöspora (Oïdium).—In the year 1926, Chapman isolated a species of *Oöspora* (*Oïdium*) from a sewer blocked by the growth of this organism. Culture of this species in a nutrient solution led to the formation of a thick film within 2 days, which contained 10 per cent fat and 50 per cent protein. The flavor and odor of the film resembled cream cheese.[1]

Out of 50 strains of *Oöspora* (*Oïdium*) *lactis* examined—10 of which produced considerable quantities of fat—Fink, Haeseler, and Schmidt[2] selected two, strains *A* and *B*, which reproduced well and produced good yields of fat. Of these two strains, strain *A* was preferred, for it was less sensitive to higher temperatures and it reproduced more uniformly.

[1] Ramsbottom, J., *Brit. Assoc. Advancement Sci.* (*Annual Report*), Sept. 10, 1936.

[2] Fink, H., G. Haeseler, und M. Schmidt, *Zeit. Spiritusind.*, **60**: 74, 76-77, 81-82 (1937); Fink, H., H. Haehn, und W. Hoerburger, *Chem. Ztg.*, **61**: 744-747 (1937).

Oöspora lactis, or, as it has been commonly designated in the past, *Oidium lactis*, is frequently found in Camembert cheese[1] and in some butters and may be a cause of spoilage of cream cheeses. It is quite resistant to heat and infection by other microorganisms. This attribute of growing in a medium that has become infected by other microorganisms and, at the same time, giving good results makes the mold especially desirable.

Optimum Conditions for Fat Production.—Since *Oöspora lactis* occurs naturally in dairy products, whey is an excellent source of raw material. Whey may be used alone or enriched with other nutrient substances. Urea, ammonium sulphate, or ammonium acetate are good sources of nitrogen, while primary potassium phosphate (KH_2PO_4) and magnesium sulphate are excellent sources of potassium, phosphate, magnesium, and sulphur.

As an illustration of the nature of the nutrient medium and the results obtained when *Oöspora lactis* is grown in Jena flasks, the following experiment is quoted:[2] The nutrient solution contained 2 liters of whey, 2 g. of ammonium sulphate, 1 g. of primary potassium phosphate, and 0.5 g. of magnesium sulphate. In each of a series of Jena flasks, 250-cc. portions were placed. Data for the experiment are shown in Table 63.

An examination of this table shows that under the conditions of the experiment the maximum yield of crude fat was obtained in 6 days.

Other experiments by Fink, Haeseler, and Schmidt have indicated that the shallow pan or dish was superior to the Jena flask as a container.

The optimum temperature for fat production was 25 to 30°C.

A sugar concentration of 4 to 6 per cent was satisfactory for both strains *A* and *B*.

Using strain *A*, Fink and his coworkers obtained yields of 12.5 to 14.34 g. of crude fat within 5 days from 100 g. of sugar in whey enriched with ammonium sulphate, potassium chloride, and magnesium sulphate. These yields, according to Fink and his associates, were better than those obtained by Geffers[3] from pure wheys, calculated on the sugar utilized, when using strains of *Oöspora wallroth*, strains similar to *Oöspora lactis*, and were secured in a shorter period of time. However, Geffers obtained yields as high as 50 per cent of the dry weight of *Oöspora wallroth*, when using lactose.

Production from Straw and Oat Hulls.—A German process for the production of fat by *Oöspora (Oidium) lactis* from the sugars derived from

[1] Hucker, G. W., "Dairy Bacteriology," 2d ed., John Wiley & Sons, Inc., New York, 1935.

[2] Fink, Hahn, und Hoffburger, *loc. cit.*

[3] Geffers, H., *Arch. Mikrobiol.*, 8: 60–98 (1937).

straw (by a modified Scholler process) and from oat hulls has been described by Balls.[1] Two types of apparatus were used. One of these consisted of a rotating drum, made by stretching canvas over a wooden frame about 4 ft. in diameter, which was open at the ends near the central shaft. The nutrient solution was placed on the inside of the drum. In order to inoculate the medium, the inside surface of the canvas was sprayed or painted with a culture of *O. lactis*. The drum was rotated about two times a minute during operation. The second type of apparatus consisted of a row of sheets of canvas hung about 3 in. apart on a wooden frame. One side of the canvas was painted with a culture of the

TABLE 63.—DATA ON FAT PRODUCTION BY *Oöspora lactis*[1]

Age, days	Yield in dry material, grams	Nitrogen, per cent of dry substance	Crude protein extraction, per cent of dry substance	Crude fat, per cent of dry substance	Total yield of crude fat, grams	pH of the nutrient solution
2	1.777	5.40	33.75	7.5	0.133	5.0
3	3.238	3.32	20.75	10.8	0.349	(6.5)
4	3.968	3.15	19.69	13.4	0.531	5.0
5	4.771	2.98	18.62	16.7	0.796	5.0
6	5.729	2.81	17.56	22.5	1.290	7.1
7	6.147	2.76	17.25	22.0	1.352	7.7
12	5.886	2.85	17.81	19.6	1.152	8.3
16	5.710	3.15	16.69	16.6	0.948	8.1

[1] FINK, H., H. HAEHN, und W. HOERBURGER, *Chem. Ztg.*, **61**: 744–747 (1937).

organism. Nutrient solution was pumped through nozzles onto the other side of the canvas and trickled down to a tray below. It was pumped continuously from the trays to the canvas until the sugar was nearly consumed. Filtered air was circulated between the canvas sheets. The process was complete in about 5 days at room temperature. The yield was said to be about 20 per cent of fat on a dry basis. For further details, the reader is referred to Balls' report.

Fat Production by Rhodotorula Gracilis.—The production of fat from *Rhodotorula gracilis* has been described by Enebo and associates.[2,3] Fat contents of 50 to 60 per cent (on a dry basis) were obtained in noncon-

[1] BALLS, A. K., *Fiat Final Report* No. 371, Oct. 10, 1945.

[2] ENEBO, L., M. ELANDER, F. BERG, H. LUNDIN, R. NILSSON, and K. MYRBÄCH, *Iva*, **6**: 1 (1944).

[3] ENEBO, L., L. G. ANDERSON, and H. LUNDIN, *Arch. Biochem.*, **11** (No. 3): 383 (1946).

tinuous cultivation with about 51 liters of substrate. The fat coefficient (number of grams of newly formed fat per 100 g. of sugar consumed) was 16 to 18; the generation time, 15 to 20 hr.; and the protein content, 12 to 13 per cent, under these conditions.

Conditions of Production.—The medium used by Enebo and his fellow workers[1] in most of their experiments had the following composition:

Constituent	Grams/liter
Invert sugar	40.0
$(NH_4)_2SO_4$	1.0
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	1.0
NaCl	0.5
$CaCl_2 \cdot 6H_2O$	0.5
$FeCl_3 \cdot 6H_2O$	0.005
Beer wort (20 per cent)	25 ml.
pH (with H_2SO_4) to	4.5

The yeast was grown in a cylinder of acid-resistant steel, which was 25 cm. in diameter and 225 cm. high, and which was provided with an aerating device at the bottom and a rotating foam-suppressor.

The temperature of incubation was 27 to 29°C.

Observations.—In one typical experiment, the time of cultivation was 68 hr.; the volume of nutrient solution, 47 liters; the volume of the seed culture, 5.5 liters containing 46.0 g. of yeast on a dry basis; the newly formed yeast (dry substances), 547 g.; the added sugar, 1,880 g.; the yield of yeast, 29.1 per cent; the content of crude fat in dry yeast substance, 56.6 per cent; the newly formed fat, 326 g.; the fat coefficient, 17.3; the degree of reproduction, 12.9; the number of generations, 3.69; the generation time, 18.4 hr.; the nitrogen content of the yeast, 2.10 per cent; and the ash content of the yeast, 3.5 per cent.

Figure 47 shows the relationship between sugar content, quantity of yeast, and fat content as functions of time.

Fat Production by a Soil Yeast.—Starkey[2] has described lipid production by a yeast isolated from the soil, which appeared to be closely related to *Torulopsis lipofera*. He found that the lipid content of the cells was largest when the yeasts were grown on a nitrogen-deficient carbohydrate medium. Large amounts of lipid were produced in an aerated solution that contained 3 per cent glucose, 0.05 per cent $(NH_4)_2SO_4$, and 0.01 per cent yeast extract. The conversion of glucose to lipid varied from 10 to 14 per cent; the lipid content of cells (on a dry basis), from 50 to 63 per cent.

[1] Enebo, L., M. Elander, F. Berg, H. Lundin, R. Nilsson and K. Myrbäch, *Ivg.* **6**: 1 (1944).

[2] Starkey, R. L., *Jour. Bact.*, **51**: 33 (1946).

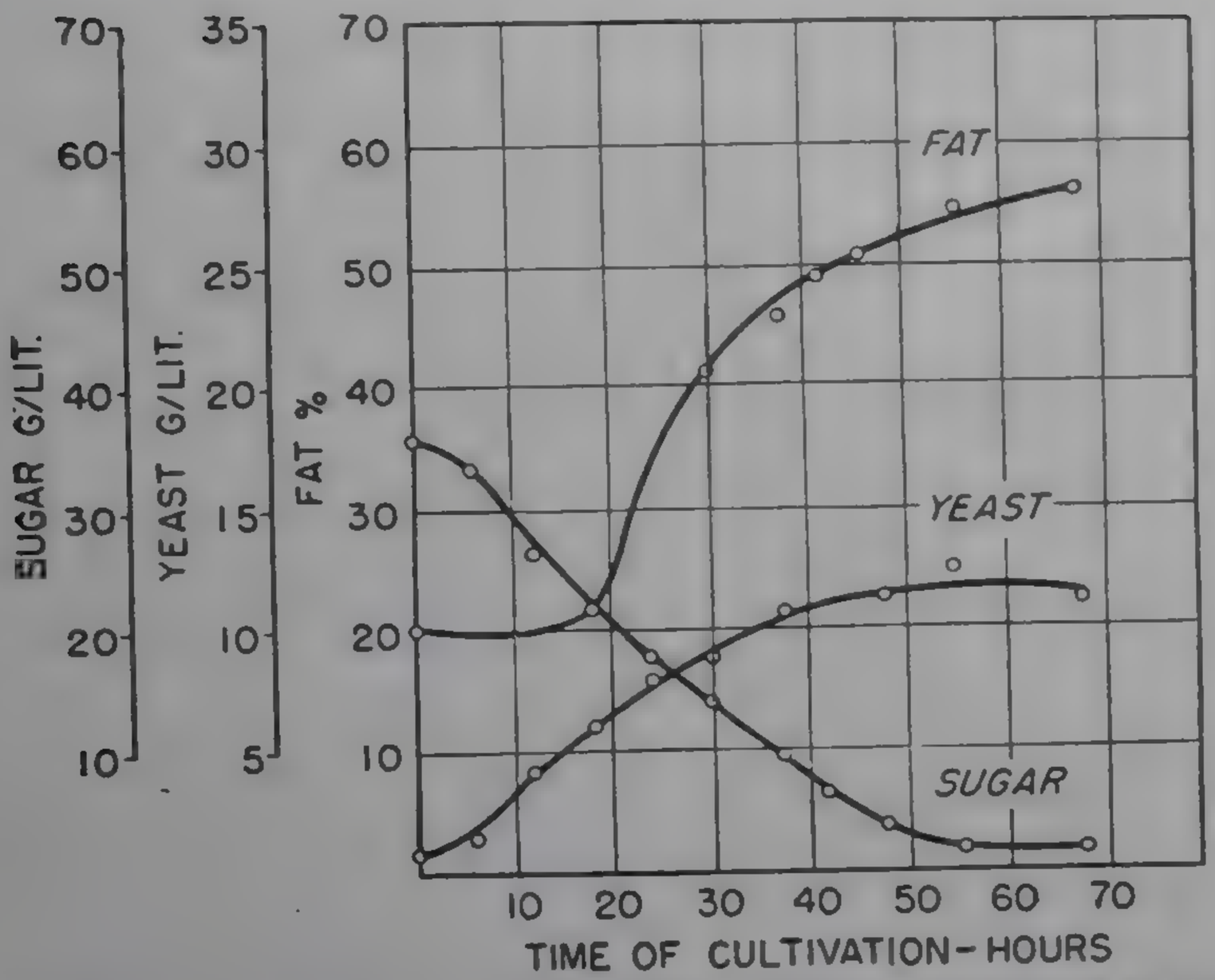


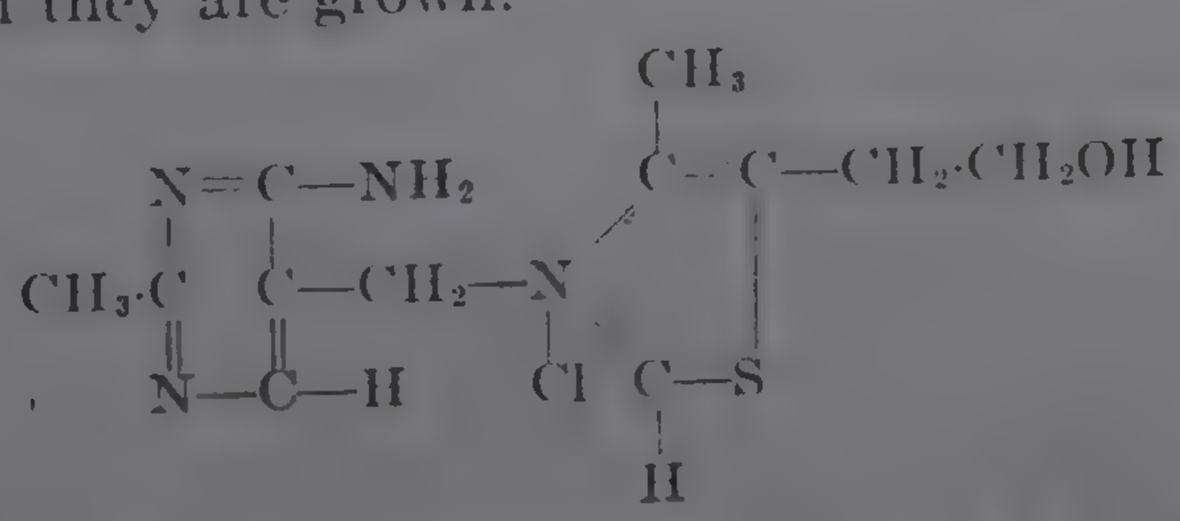
FIG. 47.—Sugar content, quantity of yeast, and fat content as functions of time. [*Courtesy of L. Enebo, L. G. Anderson, and H. Lundin, Arch. Biochem.*, **11** (No. 3): 383 (1946).]

THE VITAMINS OF YEAST

Vitamin production in yeasts varies qualitatively and quantitatively, as has already been indicated in the discussion on food yeasts. Although thiamin, riboflavin, nicotinic acid, pantothenic acid, biotin, pyridoxine, folic acid, and *p*-aminobenzoic acid are produced by yeasts in general, many strains form little or none of some of these vitamins. However, certain strains of yeasts are particularly good sources of thiamin, riboflavin, or others of the vitamins when propagated under optimum conditions.

Millions of pounds of yeast, especially brewers' yeast, have been used to enrich foods and in pharmaceutical products. Huge amounts have been employed in livestock feed.

Vitamin B_1.—Vitamin B_1 (thiamin, or aneurin), the antiberiberi vitamin, whose structural formula follows,[1] is produced in varying amounts by different yeasts, depending largely upon the nature of the medium in which they are grown.



[1] ADDINALL, C. R., "The Story of Vitamin B_1" Merck & Co., Inc., Rahway, N.J., 1937.

According to the investigations of Pavcek, Peterson, and Elvehjem, the vitamin B_1 content of most of the yeasts grown on the same medium under similar conditions was approximately the same. The vitamin B_1 content varied widely on different media, however.

The vitamin B_1 content of the yeasts listed in the following table was approximately 10 I.U. (International Units) per g. of dry yeast for the grain medium, with the exception of *Endomyces vernalis*, which yielded approximately 7 I.U. per g. For the molasses-salts medium, the vitamin B_1 content varied from 3 to 4 I.U. (*E. vernalis*, excepted); for the glucose-salts medium, the vitamin B_1 content varied from 2.5 to 3.3 I.U. (omitting *E. vernalis*).

TABLE 61.—APPROXIMATE AMOUNT OF VITAMIN B_1 PER GRAM DRY YEAST[1]

	Grain medium, I.U.	Molasses-salts medium, I.U.	Glucose-salts medium, I.U.
Baker's yeast A	10	<3	3
Baker's yeast B	10	3	<3.3
Brewer's yeast A	10	<3	<3.3
Brewer's yeast A (medium stored)	10		
Torulopsis lipo	10		
Willia anomala	10	<3	<2.5
Endomyces vernalis	10	4	<3.3
	7	5	7

[1] Data from my investigations. Yeast and the extracts.

The superiority of the grain medium from the point of view of vitamin B_1 production is evident. Both the grain and the molasses media contained vitamin B_1 before inoculation with yeast, while the glucose-salts medium contained none.

The indications are that yeast will abstract vitamin B_1 from the medium, when it is thus available, in preference to synthesizing it.[1] The addition of vitamin B_1 crystals, yeast concentrates of B_1, nucleic acid, or liver extract increased the yields of the vitamin produced by Baker's yeast, strain *B*, from the glucose-salts medium. (Nucleic acid, which contains no vitamin B_1, was used because it was believed that it might function as a precursor of the vitamin.[2])

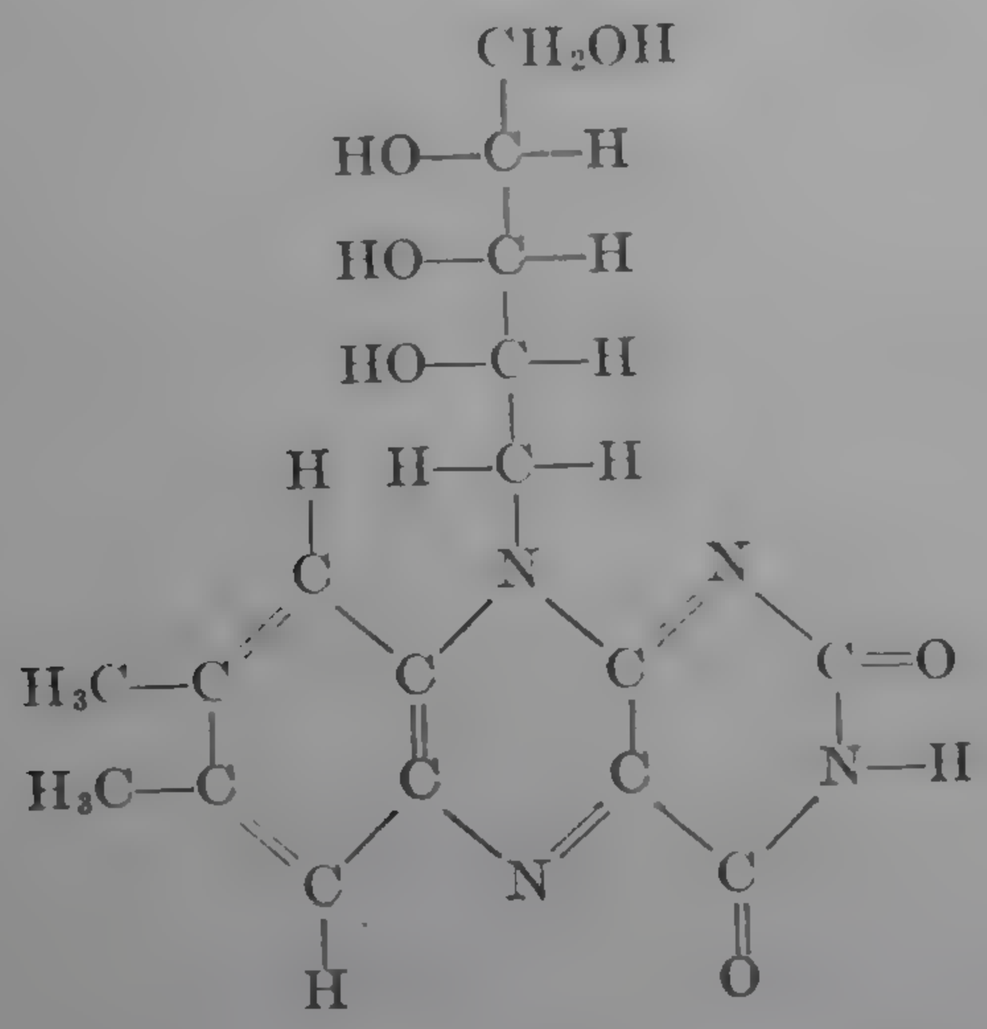
Apparently yeast is able to resynthesize vitamin B_1 from its decomposition products, for the destruction of the vitamin in grain medium by prolonged heating at pH 6 did not decrease the yield of the vitamin.[2]

[1] Peterson, A. M., Pavcek, *Ibid.*, **13**: 37 (No. 10, 1938).

[2] Peterson, P. L., W. H. Peterson, and C. A. Elvehjem: *Ind. Eng. Chem.*, **29**: 536

The vitamin B_1 potency of yeast is increased when aeration is not used, but the yield of yeast is small under such conditions.

Riboflavin Synthesis by Yeasts.—Riboflavin (vitamin B_2, vitamin G, lactoflavin) has the empirical formula $C_{17}H_{20}N_4O_6$ and the structural formula indicated below:



When in the form of a crystalline yellow-orange powder, it is bitter in taste and practically odorless.

Rogosa[1] has shown that lactose-fermenting yeasts have the ability to synthesize riboflavin when grown on a riboflavin-free medium. The results of some of his findings are summarized in Table 65 which follows.

TABLE 65.—QUANTITY OF RIBOFLAVIN SYNTHESIZED BY SOME LACTOSE-FERMENTING YEASTS[1]

Culture	Riboflavin synthesized, γ/ml. of culture
Saccharomyces anamensis No. 145	0.08
Type F No. 93	0.08
Monilia pseudotropicalis (Castellani) No. 32	0.06
Mycotorula lactis No. 130	0.08
Saccharomyces lactis No. 131	0.09
Torulopsis kefir No. 149	0.10
Zygosaccharomyces lactis No. 90	0.06
Z. lactis No. 27	0.10
Torula sphaerica No. 13	0.09
T. lactosa No. 168	0.11
T. cremoris No. 2	0.06
Saccharomyces fragilis No. 15	0.09

[1] ROGOSA, M., *Jour. Bact.*, **45** (No. 5): 459–460 (1942).

[1] ROGOSA, M., *Jour. Bact.*, **45** (No. 5): 459–460 (1942).

Production by Ashbya gossypii.—The formation of riboflavin by *Ashbya gossypii* was recognized by Guilliermond, Fontaine, and Raffy.[1] Production of this vitamin by a variant of *A. gossypii* has been studied by Wickerham and his associates.[2] In preliminary experiments, they used two media. One of these contained 0.3 per cent powdered yeast extract, 0.5 per cent peptone, and 2 per cent cerelose. The pH of this medium was 6.8 to 7.0. The second medium was of the same composition except that it contained in addition 0.3 per cent of Stimulflav, a commercial preparation of dried distillers' solubles. The pH of the latter medium was adjusted with KOH to 6.8 to 7.0.

Aeration, obtained by passing air through the medium or by agitation on a Ross-Kershaw shaker, increased the yields of riboflavin over unaerated cultures. An aeration rate of about 75 ml. of air per 400 ml. of medium per minute appeared to be optimum. Incubation was at 26 to 28°C. Foaming was prevented by the use of 3 drops per culture of a 1:1,000 concentration of octadecanol in lard oil.

Yields of 381 γ of riboflavin per milliter were obtained in 8 days from *A. gossypii*, NRRL Y-1056, in a 2 per cent cerelose-yeast extract medium aerated at the rate of 75 ml. of air per minute per culture. The yield of riboflavin was increased from about 70 γ per ml. to 120 to 130 γ per ml. in 4 days by the addition of 0.3 per cent of Stimuflav and 0.1 per cent of calcium carbonate to the yeast extract medium and by aeration at the rate of 75 ml. of air per minute per culture.

Wickerham and his associates found that sucrose or maltose (but not lactose) could be substituted for cerelose; that sugar concentrations greater than 2 per cent gave poorer yields; and that it was not necessary to remove iron from the medium in order to obtain good yields.

Production by Eremothecium ashbyii.—Riboflavin is produced industrially from *Eremothecium ashbyii* a yeast-like organism belonging to the Ascomycetes. The latter was described by Guilliermond (1935), who, together with Fontaine and Raffy (1935), noted that it produced a yellow pigment related to the flavin group. Mirimanoff and Raffy (1938) extracted the crystalline flavin from *E. ashbyii*. It was found by Schopfer (1944) that *E. ashbyii* required biotin, inositol, thiamin, and some constituents of peptone for growth and that riboflavin production was stimulated by peptone.

Riboflavin production has been described in patents. In one method described by Rudert (1945), it is produced by *E. ashbyii* from sub-

[1] GUILLIERMOND, A., M. FONTAINE, and A. RAFFY, *Compt. rend. acad. sci. Paris.*, **201**: 1077 (1935).

[2] WICKERHAM, L. J., M. H. FLICKINGER, and R. M. JOHNSTON, *Arch. Biochem.*, **9**: 95 (1946).

stantially carbohydrate-free media. Based on the total weight of nutrients, the medium contains 10 to 90 per cent of proteinaceous mate- rial, a metabolizable lipid, and nutrients such as peptone or a combina- tion of salts (0.05 per cent KH_2PO_4, 0.07 per cent $MgSO_4 \cdot 7H_2O$, 0.10 per cent NaCl, and 0.001 per cent $FeSO_4 \cdot 7H_2O$). Examples of the media and lipids used, and yields obtained, are shown in the following table:

TABLE 66a.—RIBOFLAVIN PRODUCTION FROM DIFFERENT MEDIA[1]

Lipid source	Grams/ 100 ml.	Peptone 2.4 g./100 ml. (micrograms/ml.)	Salts and egg albumen (0.6 g./100 ml.) (micrograms/ml.)	Initial pH	Final pH
Corn oil	0.6	...	178	6.5	7.1
Mazola oil	0.6	...	113	6.5	
Olive oil	0.6	110	...	6.5	7.1
Peanut oil	0.6	...	107	6.5	6.7
Cocoa oil	0.6	134	...	6.5	7.1

[1] RUDERT, F. J., U.S. Patent 2,374,503, Apr. 24, 1945.

In carrying out production, the following procedure is illustrative of Rudert's invention: The media are adjusted to an initial pH of 5.5 to 7.5 and dispensed in containers to give a depth of 0.5 in., sterilized at 20 lb. pressure for 45 min., cooled to 30°C., and inoculated with 0.7 per cent of an active culture of *E. ashbyii*. During production the temperature is maintained between 20 and 34°C. and the medium is aerated with 1.5 to 2 cu. ft. of sterile air per minute per square foot of mash surface. At the end of 50 to 90 hr. the conversion is complete and the final pH is usually 6.5 to 7.2. The dried residues contain 200 to 6,000 micrograms per g. of riboflavin.

In a patented method, described by Piersma (1946), *E. ashbyii* is grown on a medium containing animal proteinaceous material, a carbo- hydrate and malt extract. The proteinaceous material may be liver, pancreas, spleen, lung, liver, or other substance of related nature. The carbohydrate source may be glucose, maltose, sucrose, molasses, corn syrup, etc., or a combination of several of these. The concentration employed may be 1 to 5 per cent proteinaceous material, 0.25 to 1.5 per cent carbohydrate, and 0.5 to 5 per cent of malt extract (optimum 1.5 to 2.0 per cent). The pH of the medium is initially 5.5, and the temperature 27 to 30°C. Oxygen is supplied by aeration. The yield of riboflavin is generally 150 to 500 γ per ml. of medium; for example, in a mash contain- ing 4 per cent liver cake (residue from aqueous extract of liver), 0.5 per

cent sucrose, and 1.75 per cent malt extract, the yield of riboflavin was 304 γ per ml. in 88 hr.

Production by Candida Species.—The production of riboflavin by species of the genus *Candida* has been studied by Burkholder (1943, 1944), Tanner, Vojnovich, and Van Lanen (1945); Tanner and Van Lanen (1947); and others.

Burkholder (1943) found that *C. guilliermondia* grew and produced riboflavin satisfactorily in media containing dextrose, mannose, levulose, or sucrose. Asparagine and glycine were suitable and relatively inexpensive sources of nitrogen for riboflavin production.

Burkholder (1944) patented a process for producing riboflavin from *C. guilliermondia* (A.T.C.C. 9058). A medium of the following composition proved to be "highly satisfactory":

	Grams/ Liter		Parts per Million
KH_2PO_4	0.5	Boron[1]	0.01
$MgSO_4 \cdot 7H_2O$	0.5	Manganese[1]	0.01
$CaCl_2 \cdot 2H_2O$	0.3	Zinc[1]	0.07
$(NH_4)_2SO_4$	2.0	Copper[1]	0.01
KI	0.1	Molybdenum[1]	0.01
Asparagine	2.0	Iron[1]	0.01
Dextrose	20.0	Biotin[2]	1 microgram

[1] Supplied as chlorides or soluble salts.

[2] Supplied as methyl ester or crude concentrate.

Most suitable results were obtained when the pH was adjusted to 5.0 to 6.0, although the range of 5 to 7 was satisfactory. The temperature was 30°C. Increased yields of riboflavin were obtained by adding small amounts of sterile potassium cyanide or cyanide gas to the medium after vigorous fermentation was obtained, usually after 24 hr. The fermentation time was 6 to 7 days.

Tanner and Van Lanen[1] have patented a method for producing riboflavin from *Candida flareri*. The method briefly consists of growing under aerobic conditions at 30°C. for about 7 days *C. flareri*, or other suitable *Candida* species, in a medium containing a fermentable sugar, an assimilable source of nitrogen, non-iron inorganic salts, biotin, and less than 10.3 micrograms of iron per 100 ml.

The preferred species of *Candida* are *C. flareri* and *C. guilliermondia*, but other suitable species may be used.

It is essential to use great care in controlling the iron content of the medium, for iron exerts a critical influence on the yield. A low iron content may be obtained in several ways, among which are the following: (1)

[1] TANNER, F. W., JR., and J. M. VAN LANEN, U.S. Patent 2,424,003, July 15, 1947.

by the treatment of the medium by its passage through an ion exchange apparatus for the removal of iron (for example, with the use of Nalcite MX, Nalcite iron remover, or Nalcite AX[1]); (2) by the selection of the ingredients of known low iron content; or (3) by the treatment of the medium with chloroform and 8-hydroxoquinoline. In the latter procedure, the medium (about 200 ml. of the basal) is shaken in a separatory funnel with a mixture of 10 mg. of 8-hydroxoquinoline in about 2 ml. of chloroform. The mixture is allowed to stand until the chloroform separates out, after which the latter is withdrawn. Additional chloroform is added, the mixture shaken and allowed to settle, and then the chloroform separated out. This procedure is repeated, 8-hydroxoquinoline being added in alternative extractions, until the chloroform layer that settles out appears to be colorless, which indicates that not more than 0.3 microgram of iron per 100 ml. remains in the medium.

Various media may be used as long as they conform to the general specifications outlined above and contain less than 10.3 micrograms of iron per 100 ml. Tanner and Van Lanen, in several experiments, used a basal medium that contained dissolved in 200 ml. of water, 40 g. of glucose, 2.0 g. of asparagine, 2.0 g. of urea, 0.5 g. of KH_2PO_4, 0.5 g. of $MgSO_4 \cdot 7H_2O$, and 1.0 microgram of biotin (free acid). This basal medium was treated with chloroform and 8-hydroxoquinoline to remove most of the iron and made up to 1 liter with triply distilled water in a container that would not contribute iron. One hundred–milliliter portions of the medium were dispensed in 500-ml. Erlenmeyer flasks, sterilized at 126°C. for 15 min., cooled, and inoculated with a suspension of yeast cells which had been washed previously with triply distilled water in a centrifuge to remove loosely adherent iron.

As an alternate procedure, the glucose is dissolved in 1 liter of water and then treated by passage through an ion exchange column containing Nalcite MX (or other suitable iron remover). The other ingredients mentioned above are then added, and the pH is adjusted to 5.0 (4.5 to 5.5) with NaOH or other alkali. The medium is sterilized, cooled, and inoculated as described above.

Tanner and Van Lanen state in their patent that various substitutions may be made in the basal medium. Fermentable sugars, such as arabinose, dextrose, levulose, maltose, mannitol, sucrose, or xylose may be used. Ammonium chloride, ammonium nitrate, ammonium sulphate, or ammonium phosphate may be employed as the source of inorganic nitrogen, while asparagine, glutamic acid, or hydrolyzed casein may be employed as the source of organic nitrogen. Phosphorus may be supplied as phosphoric acid, dipotassium hydrogen phosphate, or ammonium

[1] TANNER, F. W., JR., and J. M. VAN LANEN, U. S. Patent 2,424,003, July 15, 1947.